SPECIES DIFFERENCES IN THE CONJUGATION OF SOME AROMATIC ACIDS

by

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"I do not know what I may appear to the world, but to myself I seem to have been only like a boy playing on the sea-shore, and diverting myself in now and then finding a smoother pebble or a prettier shell than ordinary, whilst the great ocean of truth lay all undiscovered before me."

Sir Isaac Newton (1642-1727)
from Brewster's *Memoirs of Newton*, II. Ch27.

Abstract.

(i) Conjugation reactions and aromatic acids have been reviewed.

(ii) A series of *N*-acyltaurines (taurine conjugates) has been synthesized and their infra-red and mass spectra investigated. The five characteristic bands in the infra-red spectra, which may assist in the identification of taurine conjugates from biological sources, have been given.

(iii) The metabolism of ten [*carboxy*-^{14}C]aromatic acids has been studied in the ferret. Taurine conjugation was found with seven of the acids.

(iv) Certain aromatic acids were studied in the cat and and the dog. Taurine conjugation was detected in these species also.

(v) Syrian golden hamsters given [^{14}C]phenylacetic acid excreted an unidentified metabolite (3-14% of the dose) in urine, which corresponded on t.l.c. to 3- or 4-hydroxyphenylacetic acid.

(vi) Ferrets given [^{14}C]phenylacetic acid excreted phenylacetyl-L-(+)-glutamine, a novel metabolite in non-primates.

(vii) Two species of fruit bat given [^{14}C]benzoic acid excreted benzoyl-L-(+)-glutamic acid, a novel metabolite in vertebrates. Whilst the Indian fruit bat (*Pteropus giganteus*) did not synthesize hippuric acid, the African bat, *Epomops franqueti*, did (31%

of 24h urinary ^{14}C).

(viii) The extent of reduction and subsequent acetylation of 4-nitrophenylacetic acid has been studied in man, dog, cat, ferret, pigeon and hen. The cat (25% in 48h) and the hen (14% in 24h) were found to reduce a significant amount of the dose.

(ix) The reduction and acetylation of 4-nitrobenzoic acid was also studied in the dog, ferret and rat. Half of the 24h urinary ^{14}C in the rat appeared as reduced metabolites.

(x) It is concluded that steric factors determine the direction of conjugation of an aromatic acid and that this has a prognostic basis, unlike such physico-chemical parameters as degree of ionisation or lipid solubility.

CONTENTS

Acknowledgments

I must extend a genuine gratitude to Professor R.T. Williams for his guidance and altruism throughout the last three years, and to my colleagues Drs. Peter Millburn and Paul Hirom for their frequent consultations.

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Frank Dixon was always generous with his labelled compounds and metabolites; to him I am indebted.

Finally, I wish it to be more widely known that this work was carried out under the patronage of my wife Marianne who was also responsible for the excellent typing and production of this thesis. For the many hours of selfless labour - Thanks.

CHAPTER ONE

Introduction

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Introductory Remarks.

Homo sapiens is unique among the species for many reasons. One such reason is that he has the ability to consciously affect both the environment in which he lives and his inner or physiological environment. By harnessing the many brilliant individual scientific discoveries of the last one hundred years, man has developed, with the aid of technology, the chemical tools to relieve pain and suffering. Today, more than ever before, man's reliance on chemotherapy as a panacea is creating problems. Every year new drug-induced diseases and toxicities manifest themselves. The most outstanding example is still the "Thalidomide Tragedy", which needs no further qualification. This single incident, albeit emotive, underlines our need to thoroughly investigate the relationship between the New Technology and our *milieux intérieurs*.

We all, both actively and passively, ingest, inhale or absorb from our environment substances which are not concerned with the production of energy and the maintenance of homeostasis. These potentially harmful foreign and generally non-nutrient substances include drugs, food additives and pesticides.

The Law now requires adequate toxicological testing of many groups of new proprietary foreign compounds including medicines. The marketing organisation may itself undertake a study of the metabolism of the substance in question in a variety of sub-primate species, usually rat and dog, before extending their work to monkeys and ultimately man. The value judgements which must be made before widening the study to include human volunteers and finally marketing the compound require a prior knowledge of the biochemistry and

physiology of both the experimental animal and man before an extrapolation between the two can be made. It is on this solitary premise, academic interests aside, that we must study the species variations in the fate of foreign compounds, since any inter-species extrapolation must be based on precedents.

The advent of more sensitive non-radioactive techniques such as gas chromatography interfaced with mass spectrometry (g.c.-m.s.) would allow us to test directly on man new compounds at doses much lower than the pharmacologically effective or manifestly toxic level and still separate and identify the metabolites in the various biological fluids. However, it must always be assumed that until otherwise proven, a single molecule of a new and untried drug may be carcinogenic or otherwise latently toxic. The need for animal testing will therefore always stand, and a requirement for the best laboratory models for screening foreign compounds will surely exist. New and improved methods of testing, both *in vivo* and *in vitro*, can only develop from a more flexible approach to the problem which may uncover cheaper and more reliable species options.

General Aspects of Foreign Compound Metabolism.

When compounds which are normally considered as foreign to the organism (xenobiotics) enter the body, the majority of them are metabolised to some extent and thus transformed into other substances. The metabolic pathways of such foreign compounds are often referred to as detoxication mechanisms (Williams, 1959). The commonly accepted rationale for the existence of detoxication mechanisms is the need to turn non-

polar, fat-soluble xenobiotics into water-soluble metabolites that may be excreted by the kidney (Peters, 1962). In this simplistic model, the glomerular filtrate formed in the Bowman's capsule contains all the plasma components except protein. When water is reabsorbed lower down the nephron there is a tubular discrimination against polar detoxication products and only the non-polar xenobiotics can diffuse back into the circulation from the urine forming in the tubule. The water-soluble drug metabolites, which it is assumed cannot penetrate the lipid environment of the tubular membrane, accumulate in the urine and are thus excreted.

The metabolism of foreign compounds has been described as a biphasic process (Williams, 1959). In the initial phase (Phase I), the compound may undergo an oxidative, reductive or hydrolytic reaction. These processes introduce into the molecule or unmask within its structure such groups as OH, NH_2, COOH or SH. The modified xenobiotic may now be a substrate for various synthetic reactions (conjugations) in the second phase (Phase II) of the detoxication process. In general, Phase II reactions involve the conjugation of the parent compound or its Phase I biotransformation products with endogenous substances such as sugars or amino acids, leading to more water-soluble and acidic products. In a minority of cases, for example acetylation of aromatic amines and methylation of catechols (discussed later), the product of conjugation is both less water-soluble and less acidic than its precursor.

The overall biphasic scheme has been represented as follows:

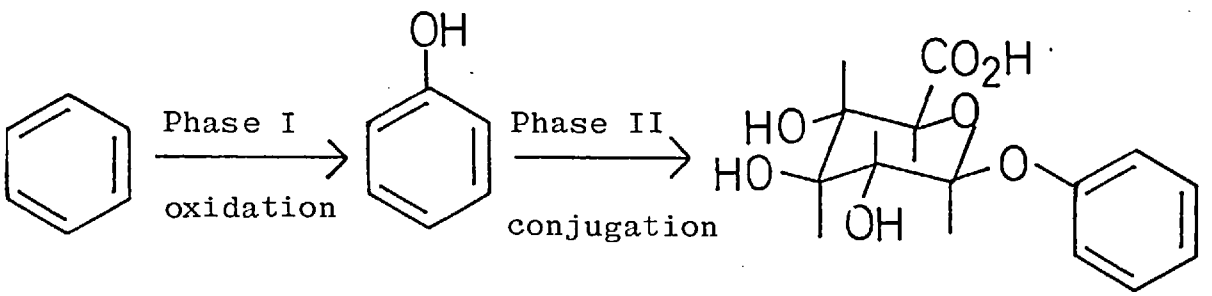
xenobiotic —Phase I enzymes→ oxidation, reduction, hydrolysis products —Phase II enzymes→ synthetic or conjugation products.

(After Williams, 1974).

The simplest and most widely used example of the biphasic nature of foreign compound metabolism and its consequences on water solubility is the metabolism of benzene, a water-insoluble neutral foreign compound (see Fig.1.1).

Fig.1.1 The Biphasic Detoxication of Benzene.

(After Williams & Millburn, 1975)



Benzene	Phenol (pKa 10)	Phenyl-β-D-glucuronide (pKa 3.4)
	0.25% ionised at pH 7.4	99.99% ionised at pH 7.4

Thus Phase I metabolism introduces into the benzene molecule a polar hydroxyl group which may be subsequently conjugated with glucuronic acid in a Phase II process giving a metabolite which is highly water-soluble and thus readily eliminated from the body.

The animal body has the ability to perform a multitude of biotransformations on foreign compounds, and the biochemical operations effected on a single administered compound

can be influenced both qualitatively and quantitatively by a number of factors which are listed below:

Species	Size of dose
Strain	Sex
Age	Stress
Chronic administration	Temperature
Route of administration	Time of day
Presence of other foreign compounds	Route of excretion (urine, bile)
Disease	Gut flora
Diet	Season
	Altitude

(After Williams, 1974)

The most significant and profound of these factors is species, and this is not surprising since species differences incorporate biochemical, physiological, morphological and anatomical differences between animals. Variation in the metabolism of foreign compounds among the species may be viewed from two main aspects: a) Variation due to differences in the rate of transformation along a common route of metabolism, and b) Variations due to differences in the actual routes of metabolism (Williams, 1964). The rate of transformation may depend on variations in the amount of an enzyme that metabolises the compound, on the amount of a natural inhibitor of the enzyme, on the activity of an enzyme reversing the reaction and on the extent of competing reactions for the same substrate (Williams, 1974).

Phase I Biotransformations.

The preponderance of oxidations, reductions and hydrolyses which have been classified as Phase I reactions are carried out by enzymes located in the endoplasmic reticulum of the hepatocyte (Fouts, 1971). Some drugs may be metabolised by the kidney and gastro-intestinal mucosa and sometimes lung and adrenal tissue and even blood cells have the ability to transform foreign compounds (Williams & Millburn, 1975). Many of the known Phase I reactions of foreign compounds can be carried out *in vitro* using disrupted preparations of liver endoplasmic reticulum in the form of vesicles (microsomes). *In vitro* studies have contributed greatly to our knowledge of the mechanisms of Phase I reactions. These mechanisms have been reviewed by Hutson (1970, 1972, 1975), and essentially comprise oxidative reactions since there are relatively few examples of metabolic reduction and hydrolysis of foreign compounds (see Table 1.1). The enzymes catalysing these microsomal oxidations have been called mono-oxygenases or mixed-function oxidases and are known to involve a carbon monoxide-binding pigment called Cytochrome P450. The role of Cytochrome P450 in drug oxygenation has been reviewed in some detail by Gillette *et al.* (1972). Mono-oxygenation of foreign compounds has a specific requirement for reduced nicotinamide adenine dinucleotide phosphate (NADPH) and molecular oxygen and may be represented by the following simplified scheme:

$$\text{Substrate-H}_2 + \text{NADPH} + \text{H}^+ + \text{O}_2 \longrightarrow \text{Substrate-HOH} + \text{NADPH}^+ + \text{H}_2\text{O}$$

The site of oxidation in the xenobiotic may be a carbon atom (aromatic and aliphatic hydroxylations, epoxidation,

Table 1.1 Examples of Phase I and Phase II reactions in Man. (After Smith, 1971).

Reaction Type		Examples
Phase I:	Oxidation	Hydroxylation
		N- and *O*-dealkylation
		Deamination
		Replacement of S by O
		Cleavage of ethers
		Oxidation of thio ethers to sulphoxides
	Reduction	Reduction of nitro and ketone groups
		Reductive cleavage of azo links
	Hydrolysis	Hydrolysis of esters and amides
Phase II:	Synthetic (conjugations)	Glucuronide synthesis
		Glycine conjugation
		Glutamine conjugation
		Mercapturic acid synthesis
		Methylation
		Acetylation
		Ethereal sulphate formation
		Thiocyanate formation

aromatic hydroxymethylation, *O*-dealkylation, *N*-dealkylation, oxidative deamination and oxidative dechlorination), a nitrogen atom (*N*-hydroxylation, *N*-oxide formation and oxidative deamination), a sulphur atom (sulphur oxidation) or a phosphorus atom (phosphorothionate oxidation, *i.e.* P=S $\longrightarrow$ P=O

and *P*-oxide formation).

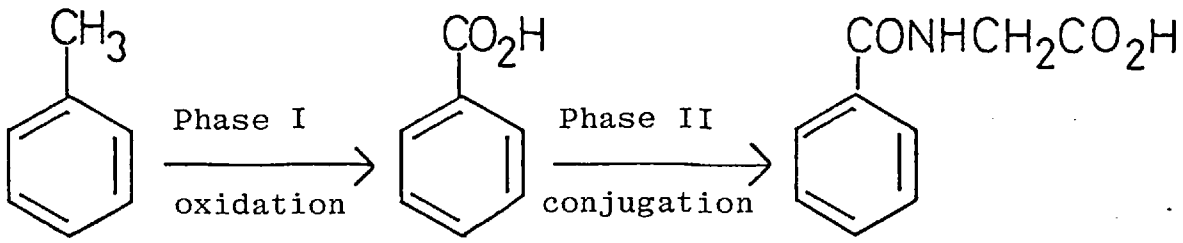
Phase I oxidations and hydrolyses can produce a carboxylic acid group and this is of particular interest since the result is that neutral foreign compounds, such as aromatic hydrocarbons and esters, can be transformed *in situ* into carboxylic acids which may then be substrates for conjugation reactions.El Masri *et al.* (1956, 1958) showed that alkylbenzenes and other related compounds when given orally to rabbits could give rise to an increase in hippuric acid excretion. Table 1.2 shows the 24h excretion of hippuric acid following the oral administration of benzoic acid, toluene, acetophenone, cinnamic acid and styrene to rabbits.

Table 1.2 Urinary Excretion of Hippuric Acid by Rabbits given Alkylbenzenes and some Related Compounds Orally. (After El Masri *et al.* 1956, 1958).

Compound	Formula	Dose (mmol/kg)	24h Hippuric Acid Excretion (% dose)
Benzoic acid	$PhCO_2H$	0.75	79
Toluene	$PhCH_3$	3.0	74
Acetophenone	$PhCOCH_3$	2.0	19
Cinnamic acid	$PhCH{:}CHCO_2H$	2.0	74
Styrene	$PhCH{:}CH_2$	5.0	40

The scheme for the biotransformation of toluene to hippuric acid is given in Fig.1.2.

Fig.1.2 The Biphasic Detoxication of Toluene.



Toluene — Benzoic acid — Hippuric acid (benzoylglycine)

Williams (1959) has also described the extensive oxidation (80-90% of dose) of 1,3-dimethylbenzene and 1,4-dimethylbenzene to the corresponding methylbenzoic acids which were almost entirely conjugated with glycine. Phase I oxidation is now known to produce carboxylic acid metabolites of many important xenobiotics including proprietary drugs such as amphetamine and propanolol and this will be dicussed in detail under "Aromatic Acids - Their Importance and Conjugation". A carboxylic acid group may also be revealed within a xenobiotic molecule by ester or amide hydrolysis and this will also be discussed under the aforementioned heading. Since carboxylic acids are fully oxidised, they cannot be produced by reductive processes, and thus Phase I reductions with respect to aromatic acids do not merit discussion here. Aromatic acids may be produced therefore by metabolic oxidation and hydrolysis of xenobiotics which may then be conjugated prior to excretion.

Phase II Biotransformations.

The rapid expansion of classical organic chemistry during the nineteenth century, especially in Germany, lead

eventually to the examination of the constituents of urine. It is logical that chemists using isolation procedures to identify urinary components should have discovered first the ultimate detoxication products or conjugates of many endogenous and exogenous chemicals. The discovery of the primary oxidative, reductive and hydrolytic reactions was to follow.

The nature and discovery of the conjugation reactions of foreign carboxylic acids, phenols and alcohols, amines and other miscellaneous groupings are shown in Tables 1.3, 1.4, 1.5 and 1.6 respectively. Information regarding even the minor conjugations, where the reaction has only been demonstrated in one species or with one substrate, has been given since this has not been fully reviewed elsewhere. Perusal of Table 1.3 reveals that foreign aromatic acids are conjugated through amide and ester linkages with endogenous substances such as amino acids and monosaccharides. The data also suggests that there exist substrate-dependent species differences in the metabolism of aromatic acids. In 1877 Jaffé found the ornithine conjugate of benzoic acid (ornithuric acid) in hen excreta and not the glycine conjugate (hippuric acid) as Keller had done in human urine in 1842, and thus this was the genesis of comparative studies in the metabolism of xenobiotics.

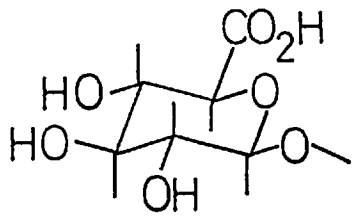
The Mechanisms of Aromatic Acid Conjugations and the Emergence of their Interspecies Variations.

In order to acquire a better insight into the species variations in the conjugation of aromatic acids, an examination of the enzymic mechanisms of these conjugations is

Table 1.3 The Nature and Discovery of the Conjugation Reactions of Foreign Carboxylic Acids.

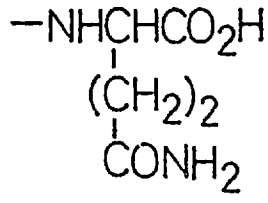
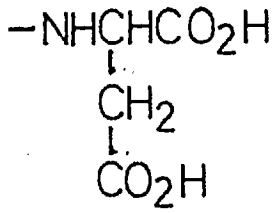
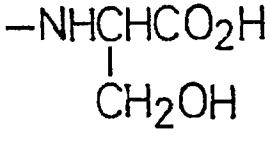
$$RCO_2H \xrightarrow{\text{conjugation}} RCO-X$$

where R = aryl or arylalkyl

	X	Author and date	Species and substrate described
Glycine	$-NHCH_2CO_2H$	Keller (1842)	man, benzoic acid
L-(+)-Ornithine	$-NHCHCO_2H$ $(CH_2)_3$ $-NH$	Jaffé (1877)	hen, benzoic acid
β-D-Glucuronic acid		Magnus-Levy (1907)	sheep, benzoic acid

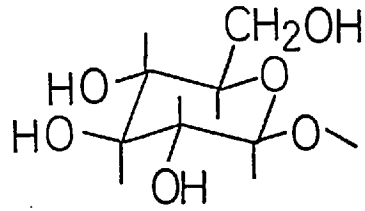
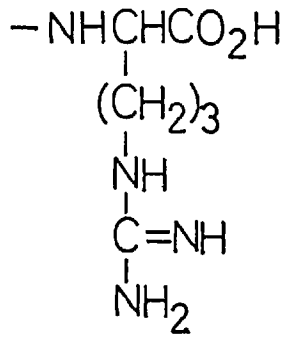
/over

Table 1.3 Contd.

L-(+)-Glutamine		Thierfelder & Sherwin (1914)	man, phenylacetic acid
L-(+)-Aspartic acid		(i) Andreae & Good (1955) (ii) Reil & Sinsheimer (1975)	(i) plants (pea seedlings), indol-3-ylacetic acid (ii) rat, *o,p'*-DDA † (*ex o,p'*-DDD)
L-(+)-Serine		Rothstein & Greenberg (1957)	rat, xanthurenic acid
Glycylglycine		Kaihara (1960)	cat, quinaldic acid

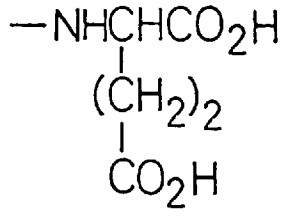
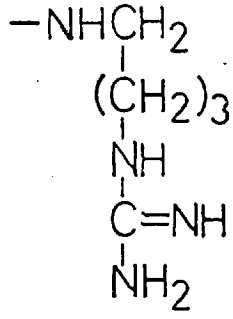
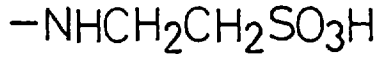
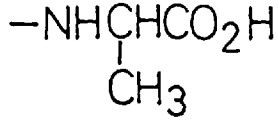
/over

Table 1.3 Contd.

Glycyltaurine		Kaihara & Price (1961)	cat, quinaldic acid
β-D-Glucose (ester)		Klambt (1962)	plants, salicylic acid (*ex* benzoic acid)
L-(+)-Arginine		Smith (1962)	spiders, 4-aminobenzoic acid 4-nitrobenzoic acid

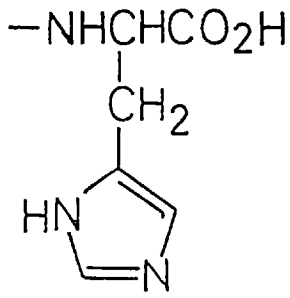
/over

Table 1.3 Contd.

L-(+)-Glutamic acid		Smith (1962)	spiders, 4-aminobenzoic acid 4-nitrobenzoic acid
Agmatine		Hitchcock & Smith (1966)	scorpions, benzoic acid 4-nitrobenzoic acid
Taurine		James, Smith & Williams (1971)	pigeon, phenylacetic acid
L-(+)-Alanine		Wallcave, Bronczyk & Gingell (1974)	mouse and hamster, DDA (*ex* DDT)

/over

Table 1.3 Contd.

L-(+)-Histidine		Smith (1976)	*Peripatus*, benzoic acid

The β-D-glucoside of indol-3-ylpropionic acid has been shown in *Bacillus megaterium* by Tabone & Tabone (1953).

The threonine conjugate, together with the serine and alanine conjugates of indol-3-ylpropionic acid, have been described in *Bacillus megaterium* by Tabone (1958).

† *o,p'*-DDA = (2-Chlorophenyl)(4'-chlorophenyl)acetic acid

o,p'-DDD = 1-(2'-Chlorophenyl)-1-(4'-chlorophenyl)-2,2-dichloroethane

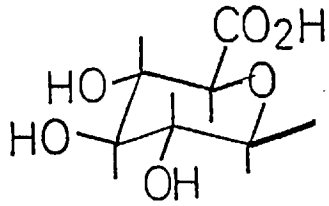
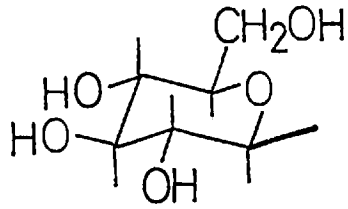
DDA = Bis(4-chlorophenyl)acetic acid

DDT = 1,1,1-Trichloro-2,2-bis(4'-chlorophenyl)ethane

Table 1.4 The Nature and Discovery of the Conjugation Reactions of Foreign Phenols and Alcohols.

$$ROH \xrightarrow{\text{conjugation}} RO-X$$

where R = aryl (phenol) or alkyl (alcohol)

X		Author and date	Species and substrate described
β-D-Glucuronic acid (ether)		Jaffé (1874)	dog, 2-nitrobenzyl alcohol (*ex* 2-nitrotoluene)
Sulphate (ethereal)	$-SO_3H$	Baumann (1876)	man, phenol
β-D-Glucose (ether)		(i) Miller (1938)	Plants (gladiolus corms), ethylene chlorohydrin
		(ii) Myers & Smith (1953)	insects (locusts), 3-aminophenol

/over

Table 1.4 Contd.

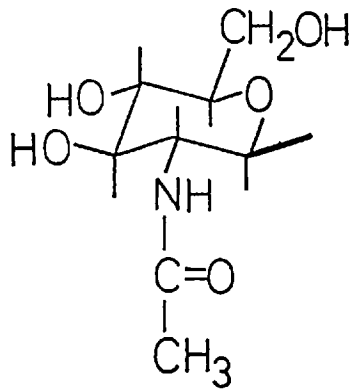
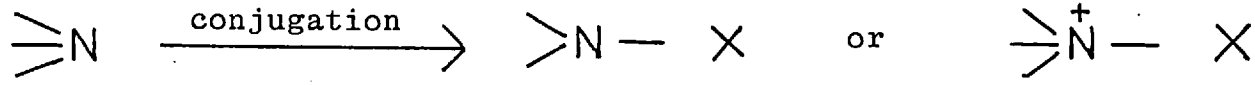
Methyl	$-CH_3$	Maclagan & Wilkinson (1954)	man, 4-hydroxy-3,5-diiodobenzoic acid (*ex* butyl ester)
Phosphate	$-PO_3H_2$	Boyland, Kinder & Manson (1961)	dog, 2-amino-1-naphthol (*ex* 2-naphthylamine)
β-*N*-Acetyl-D-glucosamine		Layne, Sheth & Kirdani (1964)	rabbit, 17-α-oestradiol-3-β-glucuronide (*ex* oestrone)

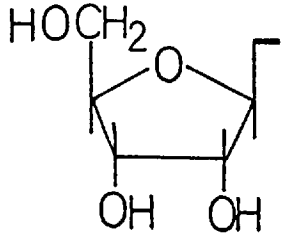
Table 1.5 The Nature and Discovery of the Conjugation Reactions of Foreign Amines.



primary, secondary, tertiary or heterocyclic amines

primary or secondary amines

tertiary or heterocyclic tertiary amines (methylation)

X		Author and date	Species and substrate described
Methyl	$-CH_3$	His (1887)	dog, pyridine
Acetyl	$-COCH_3$	Cohn (1893)	rabbit, 3- and 4-aminobenzoic acids (*ex* 3- and 4-nitrobenzaldehydes)
β-D-Ribose		Schayer (1956)	rat and mouse, imidazol-4-ylacetic acid

/over

Table 1.5 Contd.

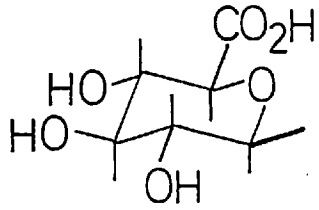
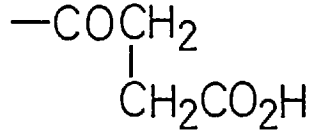
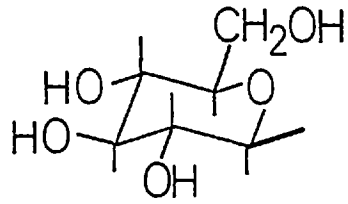
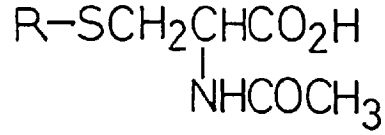
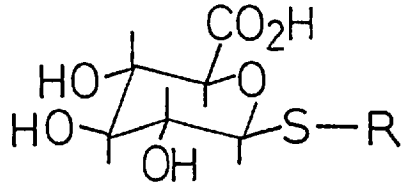
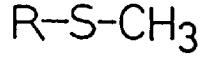
β-D-Glucuronic acid		Boyland, Manson & Orr (1957)	rabbit, 2-naphthylamine
Formyl	—CHO	Boyland & Manson (1966)	dog and rat, 2-amino-1-naphthylsulphate (*ex* 2-naphthylamine)
Succinyl		Weinstock, Parker, Lucyszyn & Intoccia (1969)	dog and rat, 1-amino-1-(4'-chlorobenzoyl) ethane (*ex N*-ethyl derivative)
β-D-Glucoside		Duggan *et al.* (1974)	dog, 3-(4-pyrimidinyl)-5-(4-pyridyl)-1,2,4-triazole

Table 1.6 The Nature and Discovery of the Conjugation Reactions Occurring at Carbon and Sulphur Atoms.

Conjugate formed		Author and date	Species and substrate described
Mercapturic acid (*N*-acetylcysteine)	 where R = alkyl or aryl	(i) Jaffé (1879) (ii) Baumann & Preusse (1879)	(i) dog, chlorobenzene and iodobenzene (ii) dog, bromobenzene
Thiocyanate (cyanide detoxication)		Lang (1894)	dog, cyanide
S-β-D-Glucuronide		Clapp (1956)	dog, 2-mercaptobenzothiazole
S-Methyl	 where R = alkyl or aryl	Sarcione & Sokal (1958)	rat, thiouracil

required. The best studied conjugation mechanism is that of glucuronidation, much of our understanding of which has arisen from *in vitro* work with phenolic substances such as 2- and 4-aminophenol. The conjugating agent in glucuronidation is now known to be uridine-5'-diphospho-D-glucuronic acid (UDP-glucuronic acid,see Fig.1.3) but this was uncertain until Storey & Dutton (1955) found that homogenates fortified with UDP-glucuronic acid and containing excess 2-aminophenol formed the glucuronide in proportion to the concentration of the nucleotide, which was therefore a substrate rather than a cofactor.

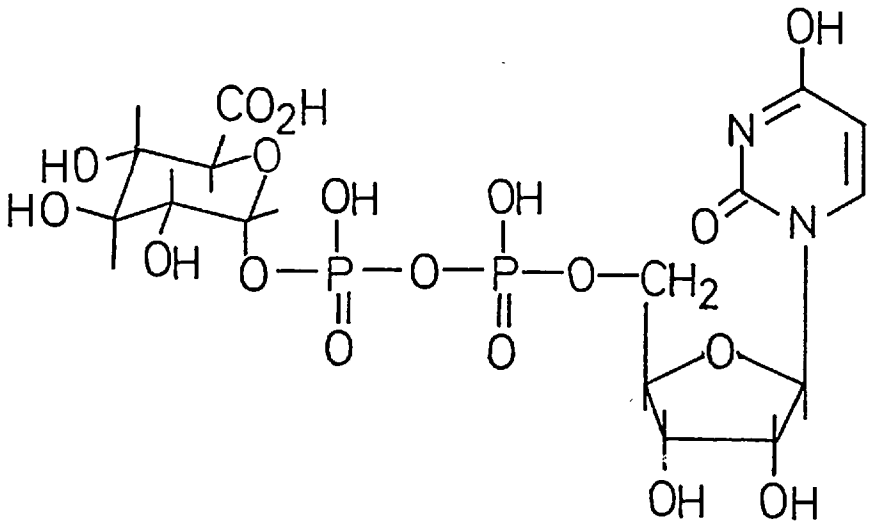


Fig.1.3 Uridine-5'-diphospho-D-glucuronic acid

The enzyme catalysing the transfer of the glucuronic acid to the substrate became known as uridine diphosphate glucuronate glucuronyltransferase (EC 2.4.1.17) but is more commonly abbreviated to UDP-glucuronyltransferase (Dutton, 1966). More recent research has intimated that a family of glucuronyl-transferases exist in liver microsomes. The use of 2-amino-phenol, 4-aminophenol, phenolphthalein, bilirubin and certain

sterols has revealed enzymes with apparently different characteristics. Gram *et al.* (1968) have examined the submicrosomal distribution of rabbit liver glucuronyltransferases. Glucuronidation of 2-aminophenol and 4-nitrophenol occurred mainly in the rough endoplasmic reticulum, whilst transferase activity towards phenolphthalein was found to be equally distributed between the rough and smooth endoplasmic reticula. Other work would also seem to indicate a multiplicity of the glucuronyltransferase system (Tomlinson & Yaffe, 1966; Zakim *et al.*, 1973; Vessey *et al.*, 1973). Some 16 distinct classes of hydroxylic, carboxylic, amino, imino and thiol function groups have been described by Smith and Williams (1966) as being conjugated with glucuronic acid *in vivo*. It is hard to conceive of a singular glucuronyltransferase enzyme with little aglycone specificity which would catalyse the transfer of glucuronic acid to such chemically varied endogenous and exogenous compounds.

With the discovery of CoA by Lipmann (1945), the enzymic mechanism of glycine and glutamine conjugation was soon elucidated, and shown to have a common basis. The carboxylic acids involved were activated, forming intermediate nucleotides with CoA, from which the acids were transferred to the amino acid under the influence of specific enzymes (Schachter & Taggart, 1953, 1954; Mahler *et al.*, 1953; Moldave & Meister, 1957). The enzyme catalysing the transfer of the foreign acyl group from CoA to glycine was located in liver mitochondria (Schachter & Taggart, 1954), and was found to be specific for glycine but relatively unspecific in its donor requirements, the aliphatic C_2 - C_{10} carboxylic-CoA derivatives serving as substrates. This

enzyme is now known as acyl-CoA:glycine *N*-acyltransferase (EC 2.3.1.13). The overall scheme for the biosynthesis of glycine conjugates is shown in Fig.1.4.

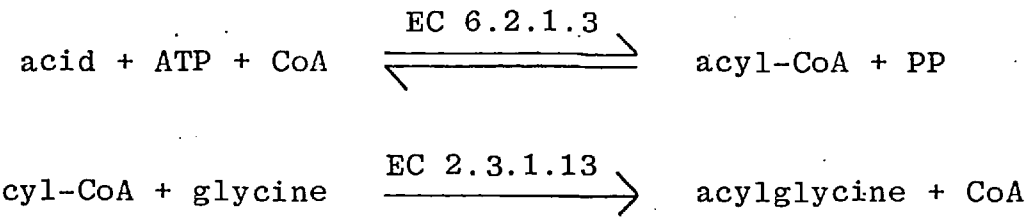


Fig.1.4 The Reaction Sequence of Glycine Conjugation.

More recently, Tishler & Goldman (1970) and Forman *et al.* (1971) have studied the *in vitro* formation of salicylglycine (salicyluric acid, the major metabolite of two of the most commonly used therapeutic agents in man, aspirin and salicylate). Like benzoic acid, salicylic acid was activated to a Coenzyme A intermediate and was then transferred to glycine by a transferase enzyme which could not be differentiated from the enzyme synthesizing hippuric acid from benzoyl-CoA in beef-liver mitochondria. The finding that isopropoxyacetic acid is excreted by rat and dog partially as its glycine conjugate (Hutson & Pickering, 1971) confirms the *in vitro* findings of Schachter & Taggart (1954) that glycine conjugation is not restricted to aromatic carboxylic acids. The location of the glycine conjugating system in mitochondria is in contrast to the majority of metabolic reactions of foreign compounds, which are generally ssociated with the endoplasmic reticulum. This may be related to the ability of mitochondria to absorb ionic substances, whereas metabolism by microsomes requires, in the main, lipid-soluble non-polar molecules (Smith & Williams, 1970). Having described

some aspects of the enzymology of aromatic acid cojugations, the possible evolution of these enzyme systems and their variation between species can now be discussed.

There is much evidence to support the theory that a large number of enzymes of diverse function in a variety of species have their origin in a single peptide about twenty amino acids long. At this early stage in evolution, variation in this primitive peptide was preparing the raw material which would give rise to different evolutionary lines of proteins (Watts, 1968). It is assumed that the modern enzymes were in existence in the most primitive organisms, and that both internal and external factors have influenced the expression of particular enzymes. The external factors influencing enzyme development are listed by Watts (1968) as food, water, salinity, gases, excercise, temperature, episomal factors (foreign genetic material such as that transferred from bacteriophage to bacterium) and noxious chemicals. These effects may be observed in both homoiothermous (warm-blooded) and poikilothermous (cold-blooded) animals, although perhaps more markedly in the latter, which have a greater dependence on the environment. Dietary changes are known not only to alter the level of the digestive enzymes but also the levels of enzymes in subsequent metabolic pathways. Marchis-Mouren *et al.* (1963) and Abdeljlil & Desneulle (1964) have demonstrated that rats fed on a high carbohydrate diet showed a two- to five-fold increase in pancreatic amylase levels, whilst the proteolytic enzyme levels were unaffected. Conversely, a high protein diet had the opposite effect. The urea cycle is particularly sensitive to dietary changes, and Shimke (1962, 1963) has shown that enzyme levels in the urea

cycle are directly proportional to the protein uptake. This was confirmed by Wergedel & Harper (1964) who showed that adaptation of the urea cycle enzymes to a high protein diet conferred protection against ammonia poisoning. Maudsley & Radwan (1968) investigated dietary influence on histidine decarboxylase at the protein synthesis level. The activity of this enzyme falls drastically during starvation, but is restored within 4-6 hours of feeding. The induction is inhibited by cyclohexamide but not by actinomycin D, showing that new protein is synthesized but the control occurs at the ribosomal level (translation and peptide bond synthesis) rather than at the nuclear level (transcription of new mRNA from existing genetic material).

Thus the changes in environment which evoke various responses from enzymes provide the modulations of natural selection which permit or reject the contribution of a new enzyme variant. Selection acts on the whole organism, but it does so by probing for the weakest link in the enzyme complement. Only those enzymes whose functions are less than essential have any scope for evolutionary experimentation (Watts, 1968). The phylogenetic development of the enzymes transforming foreign compounds should also have been subject to such selection.

The Phase I oxidising system comprising a compact microsomal electron transport chain of cytochromes and cytochrome reductases, with its single function of monooxygenation of substrates, would provide little scope for natural selection. Genetic experimentation through natural selection of one of the proteins involved in the P450 system would result in the inability to oxidise many substrates

and would thus be deleterious to the organism. However, the Phase II conjugating systems have provided much opportunity for natural selection and the influence of external factors, such as diet. The Phase II metabolising system is generally of a much greater protein complexity, with a multiplicity of enzymes of similar function, (UDP-glucuronyltransferases for different types of substrate, and *N*-acyltransferases for different amino acids, such as glycine, glutamine and taurine), occurring in both mitochondria (amino acid conjugation) and endoplasmic reticulum (glucuronidation). Unlike the P450 system, there is usually only one site available in the molecule to the conjugating system (hydroxylation of benzoic acid may occur in five sites in the molecule, and conjugation in only one), but it would appear that there are many enzymes that may interact with this single site, producing a variety of conjugates. If Watts' hypothesis of the evolutionary development of enzyme function is applied, and it is assumed that the modern conjugating enzymes were originally primitive enzymes, then both natural selection through gene mutation and extraneous influences, such as diet, could have given rise to the interspecies variations in foreign compound conjugation as they are observed today. This may have occurred through the non-expression of certain enzymes by deletion or repression of part of the genome. It is speculated that the so-called "species defects" in foreign compound metabolism have evolved for these reasons. These "defects" are mainly perturbations of Phase II reactions (see Table 1.7) and not Phase I metabolism, where the only significant abnormality is the absence of *N*-hydroxylation in the guinea pig. *N*-hydroxylation of the carcinogen 2-acetyl-

aminofluorine was not detected in guinea pigs fed on a diet containing, or injected with, this compound (Miller & Miller, 1960; Miller *et al.*, 1964). Furthermore, Irving (1964) could find no evidence for the *N*-hydroxylation of 2-acetylaminofluorine by guinea pig microsomes *in vitro*. Interestingly enough, the guinea pig is the only species which is refractory to the carcinogenic effects of 2-acetylaminofluorine. Miller & Miller (1969) consider the carcinogenesis of this compound to be mediated through the processes of *N*-hydroxylation and subsequent *O*-conjugation to produce an "active metabolite" which is responsible for the onset of neoplasia.

Table 1.7 Common Animal Species Defective in Certain Conjugations. (After Williams, 1967).

Conjugation	At low level or absent in
Glucuronide synthesis	Cat
Acetylation of aromatic amines	Dog
Mercapturic acid synthesis	Guinea pig, man, hen
Hippuric acid synthesis	Hen
Sulphate conjugation	Pig

The most interesting and profound consideration of the Phase II metabolic pathways is that of species similarities (and not differences) in the handling of xenobiotics. For example, Bridges *et al.* (1970) found that 20 of the 21 species dosed with benzoic acid excreted hippuric acid as a major urinary metabolite. The only exception was the Indian fruit bat (*Pteropus giganteus*), which excreted mainly benzoylglucuronide. This was confirmed by Bababunmi *et al.* (1973) and

later by Ette *et al.* (1974), who also showed that these bats were able to make the glycine conjugate of phenylacetic acid, and thus this metabolic defect was substrate-dependent. The fact that such varied species from primates to reptiles, and even certain insects (Smith, 1968), utilise mainly glycine for benzoic acid detoxication, and thus it is assumed also utilise a glycine *N* - acyltransferase, adds credence to the hypothesis that these enzymes in the various species had a common ancestry. Defects or abnormalities in these conjugations may have developed phylogenetically by gene mutation or under the influence of external stimuli, and thus have been compensated for by the competing reactions which already existed from the primeval ancestors. Thus, in the arachnids and certain myriapods, where glycine conjugation is not utilised for benzoic acid (Smith, 1968), arginine conjugation predominates. Benzoylarginine is then proposed to give rise to secondary metabolites such as the agmatine, glutamine and glutamic acid conjugates. Similarly, in the Indian fruit bat the glucuronic acid conjugate is the major metabolite (Bridges *et al.*,1970; Bababunmi *et al.*, 1973; Ette *et al.*, 1974). Furthermore, the domestic cat is known to be unable to form glucuronides with various substrates (Robinson & Williams, 1958). Whilst the major metabolites of phenol found in the urine of most mammalian species are phenylglucuronide and phenylsulphate (Capel *et al.*, 1972), the cat excretes phenol mainly as phenylsulphate. Closer examination of phenol metabolism in the cat by Capel *et al.*, (1974) revealed that 8% dose was excreted as phenyl dihydrogen phosphate. It would appear therefore, that phosphate conjugation of phenol was compensating for the poverty of glucu-

ronidation in this species.

In summary therefore, it is assumed that the foreign compound metabolising enzymes had a common basis prior to the radiation of the species. Deletion or repression of the more vulnerable conjugating enzymes in various species may have occurred through genetic experimentation, which today manifest themselves as species differences in xenobiotic conjugations. Pathways such as glycine and glucuronic acid conjugation have survived in most species, giving rise to overt species similarities in the conjugation of foreign compounds. For aromatic acids, more than for any other type of substrate, this is more apparent, due to the ability of the carboxyl group to react with the constellation of amino and hydroxyl functions available in the organism, which effects the manifold aromatic acid conjugations given in Table 1.3. In the light of this hypothesis, glutamine conjugation, for example, should not be viewed as being specific to primates, but originally distributed throughout much of the animal kingdom. Therefore, glutamine conjugation may be a functional possibility in some non-primate species where the synthesizing enzyme is only repressed. It is contemplated that glucuronic acid, glycine, glutamine and taurine conjugations are widespread in their species occurrence and that in-depth studies should reveal their existence. The effect of the physicochemical properties of a particular substrate on the extent of a single conjugation reaction will be discussed.

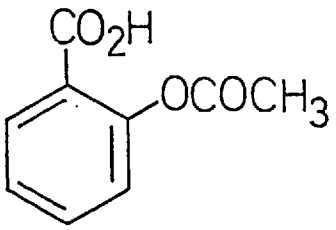
Aromatic Acids - Their Importance and Conjugation.

In this study the term "aromatic acid" is given to mean a chemical compound which is both aromatic and contains a carboxylic acid group. This definition includes a wide variety of chemical structures such as derivatives of benzoic acid, naphthoic acid, phenylacetic acid, phenoxy-acetic acid and acids with a longer side-chain or where the aromatic nucleus is heterocyclic.

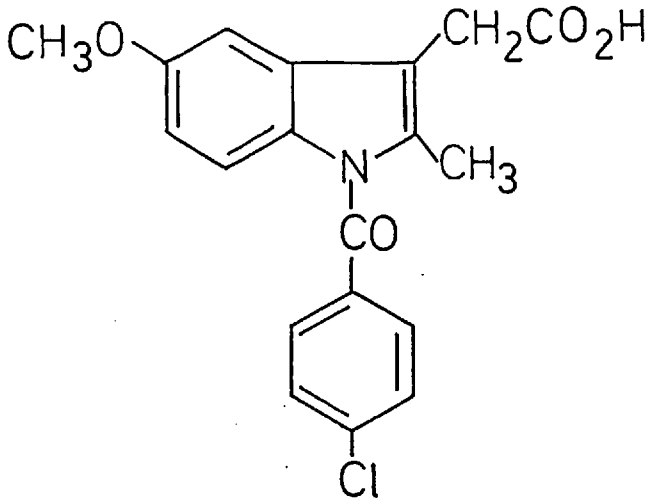
The animal organism may encounter foreign aromatic acids in two distinct ways: either as xenobiotics which are aromatic acids *per se*, or which the organism has synthesized from another xenobiotic by one of a number of processes. These steps may be classified as oxidation, hydrolysis or aromat-isation.

Many proprietary medicines are aromatic acids. In particular there are many which possess anti-pyretic, analgesic or anti-inflammatory activity and are thus used in the treatment of such conditions as rheumatism. Aspirin and indomethacin are notable in this respect, but due mainly to the gastro-intestinal haemorrhaging which these two drugs cause, they have been largely superceded by other non-steroidal anti-inflammatory agents. The chemical formulae of aspirin, indomethacin and some other aromatic acids exhibiting anti-inflammatory actions are given in Fig.1.5. Other proprietary aromatic acids include mersalyl, frusemide and ethacrynic acid (diuretics); probenecid and ethebenecid (uricosurics); nalidixic acid, sodium aminosalicylate and phthalylsulphathiazole (synthetic anti-bacterials). The structures of these drugs are shown in Fig.1.6. In order to stress the relative importance of these drugs and their effect

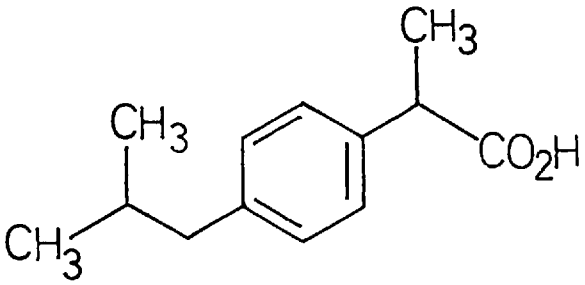
Fig.1.5 Some Non-steroidal Anti-inflammatory Aromatic Acids.



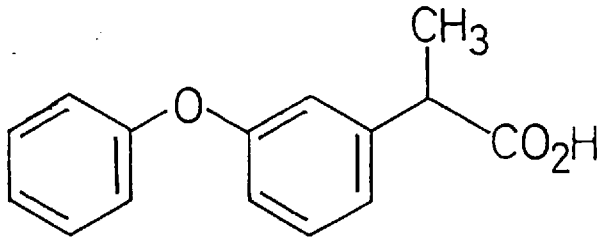
Aspirin



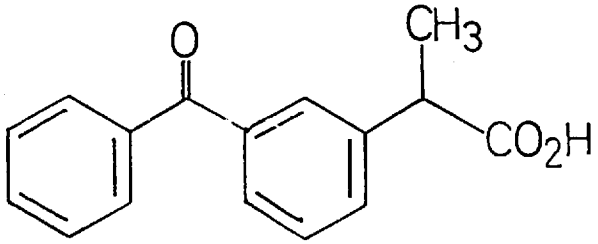
Indomethacin



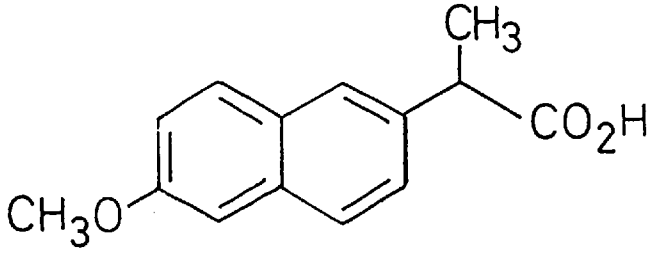
Ibuprofen



Fenoprofen



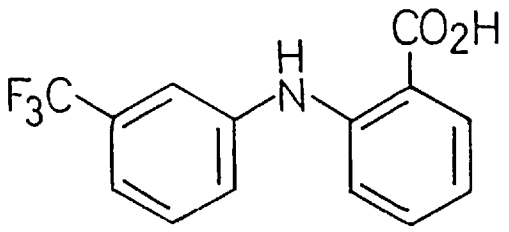
Ketoprofen



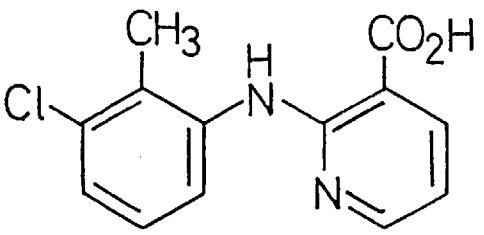
Naproxen

/over

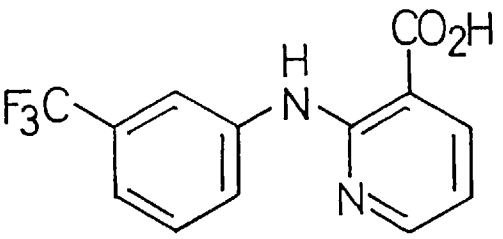
Fig.1.5 Contd.



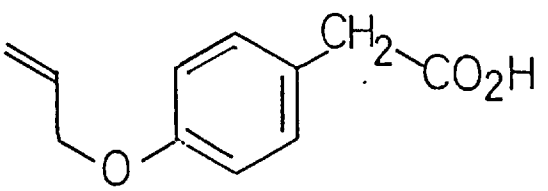
Flufenamic acid



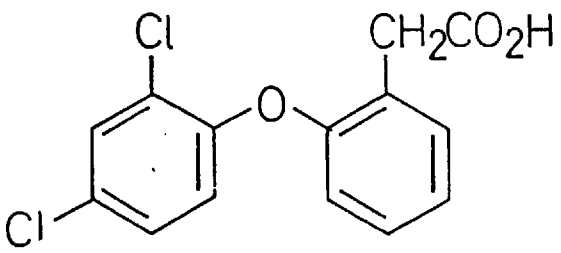
Clonixin



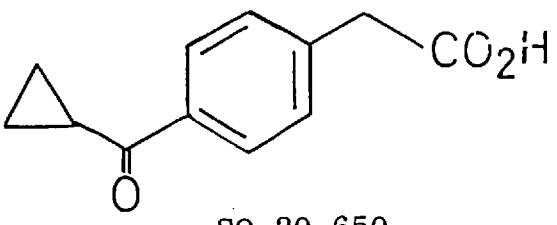
Niflumic acid



Alclofenac

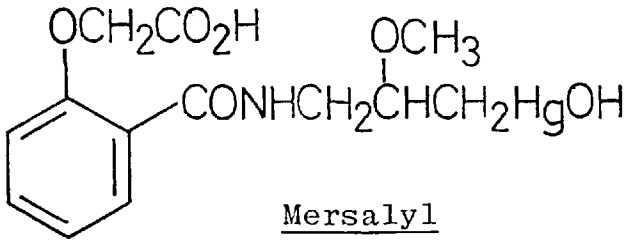


Fenclofenac

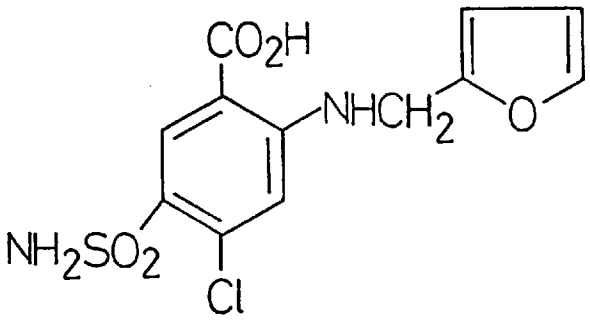


SQ 20,650

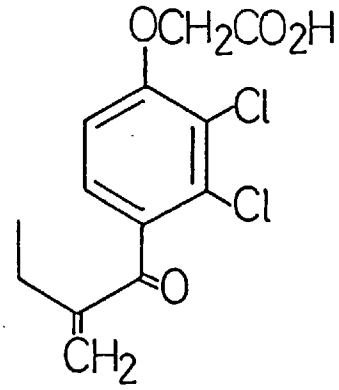
Fig.1.6 Some Aromatic Acids with Diuretic, Uricosuric or Anti-bacterial Properties.



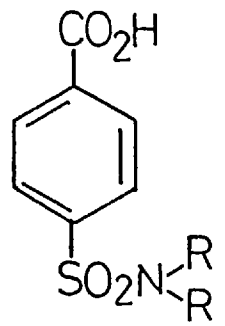
Mersalyl



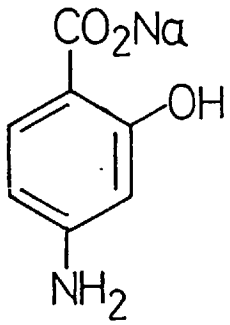
Frusemide



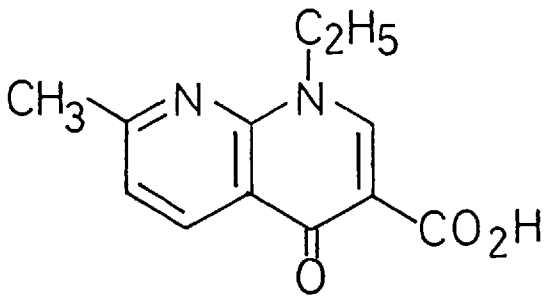
Ethacrinic acid



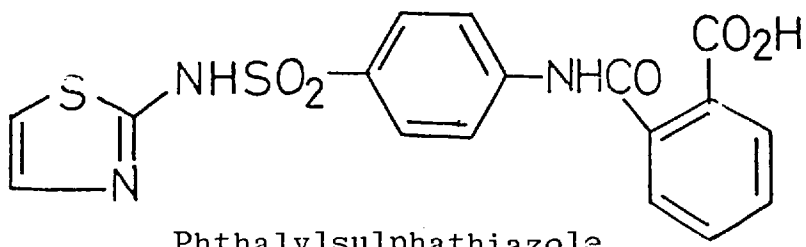
R = n-Propyl, Probenecid
R = Ethyl, Ethebenecid



Sodium aminosalicylate



Nalidixic acid



Phthalylsulphathiazole

on the environment, the 1970 and 1974 prescription figures are given for various compounds, and are shown in Tables 1.8 and 1.9 respectively. The number of reported drug-implicated fatalities has been added in Table 1.8 for interest.

The first metabolic route which may produce an aromatic acid *in vivo* is oxidation. The findings of El Masri *et al.* (1956, 1958) that rabbits fed certain alkylbenzenes excreted these compounds extensively in the form of hippuric acid has been discussed.More recently, many foreign compounds, including medicines, have been shown to be transformed in the body into aromatic acids by oxidative processes such as oxidative dechlorination, oxidative deamination, oxidative ring cleavage and oxidation of aliphatic side-chains. Thus a great diversity of chemical structures may be transformed in this way. Many of these conversions demonstrated *in vivo* during the last two decades have been listed in Tables 1.10, 1.11 and 1.12. They have been classified as transformations giving rise to arylformic acids (benzoic acids), arylacetic acids and all others (*e.g.* arylpropionic acids and aryloxy-acetic acids). Two interesting examples not included in these Tables are the oxidative biotransformations of nalidixic acid (anti-bacterial) and ibuprofen (anti-inflammatory), which are both aromatic acids and are thus metabolised to aromatic dicarboxylic acids. McChesney *et al.* (1964) found that nalidixic acid was partly converted to the 3,7-dicarboxylic acid in man (see Fig.1.7), and Adams *et al.* (1969) found that a major metabolite of ibuprofen in man was (+)-2-[4'-(2"-carboxypropyl)phenyl]propionic acid (see Fig.1.9)

It can be seen from Tables 1.10 to 1.12 that many of the aromatic acids generated by oxidative processes are substrates

Table 1.8 1970 Prescribing Figures for some Drugs which are Aromatic Acids.

Drug	EC10 Prescriptions† (thousands)	Reported drug-implicated fatalities
Aspirin	5480	146 (1964-1971)
Indomethacin	2433	72 (1964-1971)
Ibuprofen	745	10 (1969-1971)
Flufenamic acid	104	2 (1967-1971)
Mefanamic acid	390	5 (1964-1971)
Frusemide	2939	63 (1965-1971)
Mersalyl	25	10 (1964-1971)
Ethacrynic acid	25	5 (1964-1971)
Probenecid	67	9 (1964-1971)
Ethebenecid	1	0 (1968-1971)
Nalidixic acid	424	6 (1964-1971)
Phthalylsulphathiazole	135	0 (1965-1971)
Sodium aminosalicylate	88	16 (1964-1971)

† Prescriptions issued by general practitioners on Form EC10. Prescriptions in hospitals or private practice cannot be estimated, nor is it possible to estimate chemists' sales of drugs that can be obtained without prescription. (Committee on Safety of Medicines, 1976).

Table 1.9 1974 Prescribing Figures for some Drugs which are Aromatic Acids.

Drug	EC10 Prescriptions§ (thousands)
Indomethacin	3354
Naproxen	882
Ketoprofen	350
Fenoprofen	68
Ibuprofen	1864
Alclofenac	63

§ Committee on Safety of Medicines (1976).

for conjugation reactions. Not only the common conjugating agents, glycine and glucuronic acid, are used, but also glutamine, taurine, serine and alanine conjugates have been found.

A second metabolic pathway which may produce aromatic acids is hydrolysis. There are many enzymes which may hydrolyse esters. Holmes & Masters (1967) have resolved a total of 24 multiple forms of esterase activity in the guinea pig. Amides may be hydrolysed to give aromatic acids but at a much slower rate than the corresponding esters, (Mark *et al.*, 1951; Krish, 1963 *a*, *b*). The tuberculostatic agent pyrazinamide is hydrolysed to pyrazinoic acid in dog and man prior to conjugation with glycine (Weiner & Tinker, 1972; see Fig.1.9). The uricosuric agent halofenate is rapidly and quantitatively hydrolysed to the corresponding aromatic acid in rat, dog, rhesus monkey and man (Hucker *et al.*,

Table 1.10 Some Xenobiotics giving rise to Arylformic Acids (Benzoic Acids) by Oxidation *in vivo*.

Compound	Species	Acid produced	Conjugated with	Reference
Estragole (Artificial flavouring)	Rat	4-methoxybenzoic acid	glycine	Solheim & Scheline (1973)
Pronethalol (β-blocker)	Man, rabbit guinea pig and rat	2-naphthoic acid	n.c.	Bond & Howe (1967)
Fenfluramine (Anorexiant)	Man	3-trifluoromethyl benzoic acid	glycine (63-93% dose)	Bruce & Maynard (1968)
"	Rat	"	n.c.	
3,4-dichlorobenzyl -*N*-methylcarbamate (Insecticide)	Rat	3,4-dichlorobenzoic acid	glycine	Knaak & Sullivan (1968)
Ionox 100 (Anti-oxidant)	Rat	3,5-di-t-butyl-4-hydroxybenzoic acid	glucuronic acid	Wright *et al.* (1965)
Amphetamine (Anorexiant)	Dog, rabbit, guinea pig, rat and mouse	benzoic acid	glycine, glucuronic acid (mouse and guinea pig)	Dring *et al.* (1970)
Chlormezanone (Anti-convulsant)	Man	4-chlorobenzoic acid	glycine	McChesney *et al.* (1967)

/over

Table 1.10 Contd.

Bis(4-acetoxyphenyl) -cyclohexylidine methane (oestrogen)	Man	4-hydroxybenzoic acid	n.c.	Larsson (1965)
3,5 - dimethylpyrazole (Hypoglycaemic)	Rat	5-methylpyrazole-3-carboxylic acid	unidentified conjugate (15% dose)	Smith *et al.* (1965)
Metaxalone (Muscle relaxant)	Dog and man	5-(3'-methyl-5'-carboxyphenoxymethyl)-2-oxizolidinone	glucuronic acid	Bruce *et al.* (1966)
Glycodiazine (Hypoglycaemic)	Man (normal and diabetic)	2-benzenesulphonyl amido-5-carboxymethoxy pyrimidine	n.c.	Gerhards *et al.* (1964)
Seclazone (Anti-inflammatory)	Rhesus monkey and rat	5-chlorosalicylic acid	glycine and glucuronic acid	Edelson *et al.* (1973 *a*, *b*)

n.c. means not conjugated.

Table 1.11 Some Xenobiotics giving rise to Arylacetic Acids by Oxidation *in vivo*.

Compound	Species	Acid produced	Conjugated with	Reference
DDT (Insecticide)	Rat	DDA	n.c.	Jensen *et al.* (1957)
"	Mouse and hamster	"	glycine, alanine and glucuronic acid	Wallcave *et al.* (1974)
o,p'-DDT (Insecticide)	Rat	*o,p'*-DDA	glycine and serine	Feil *et al.* (1973)
Estragole (Artificial flavouring)	Rat	4-methoxyphenylacetic acid	n.c.	Solheim & Scheline (1973)
Haloperidol (Neuroleptic)	Rat	4-fluorophenylacetic acid	glycine	Braun *et al.* (1967)
Phenacetylurea (Anti-convulsant)	Rabbit	phenylacetic acid	glycine	Tatsumi *et al.* (1967)
Histamine (Gastric secretion stimulant)	Rat and mouse	imidazole-5-acetic acid	n.c.×	Snyder *et al.* (1964)
5-methoxytryptophol (Naturally occurring in pineal gland)	Rat	5-methoxyindol-3-yl acetic acid (93% dose)	n.c.	Delvigs *et al.* (1965)

/over

Table 1.11 Contd.

Mescaline (Hallucinogen)	Man	3,4,5-trimethoxy phenylacetic acid	n.c.	Charalampous *et al.* (1966)
AHR-5313 (Anti-inflammatory)	Dog	4-phenylphenylacetic acid	taurine	Turnbull *et al.* (1974)

n.c. means not conjugated.

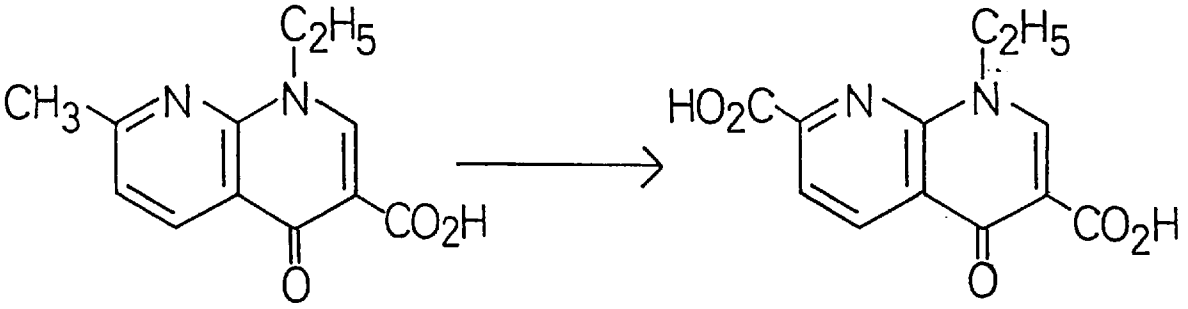
× not conjugated through carboxyl group, but forms an *N*-riboside.

Table 1.12 Some Xenobiotics giving rise to Arylpropionic Acids and Aryloxyacetic Acids by Oxidation *in vivo*.

Compound	Species	Acid produced	Conjugated with	Reference
Brompheniramine (Anti-histamine)	Man and dog	3-(4'-bromophenyl)-3-(pyrid-2'-yl)propionic acid	glycine	Bruce *et al.* (1968)
Chlorphenesin carbamate (muscle relaxant)	Rat	4-chlorophenoxyacetic acid	n.c.	Buhler (1964)
Diphenhydramine (Anti-histamine)	Rhesus monkey	Diphenylmethoxyacetic acid	glutamine	Drach & Howell (1968)
Propranolol (β-blocker)	Man and dog	1-naphthoxyacetic acid	n.c.	Walle & Gaffney (1972)
Clofexamide (CNS stimulant)	Rat	4-chlorophenoxyacetic acid	glycine	Trino *et al.* (1972)

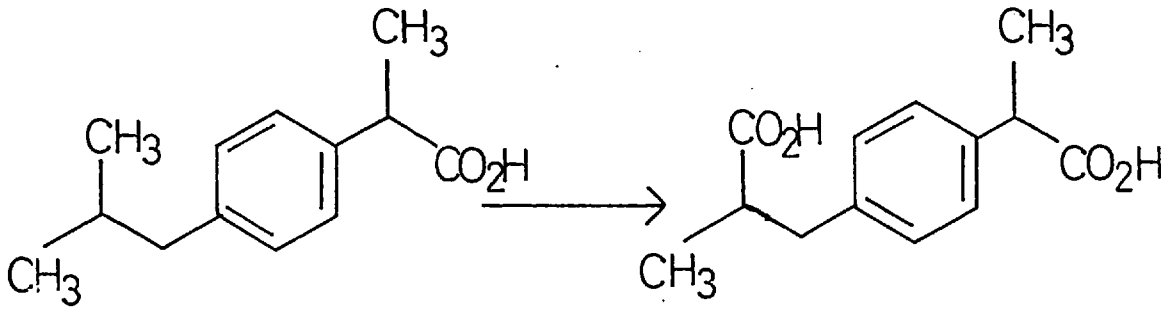
n.c. means not conjugated.

Fig.1.7 Formation of the Dicarboxylic Acid of Nalidixic Acid in Man.



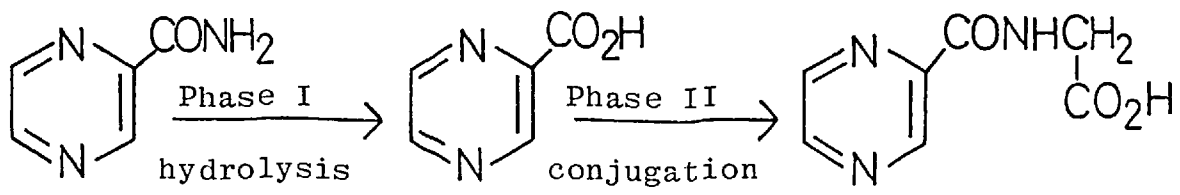
Nalidixic acid

Fig.1.8 Formation of the Dicarboxylic Acid of Ibuprofen in Man.



Ibuprofen

Fig.1.9 The Biphasic Biotransformation of Pyrazinamide in Man and Dog.



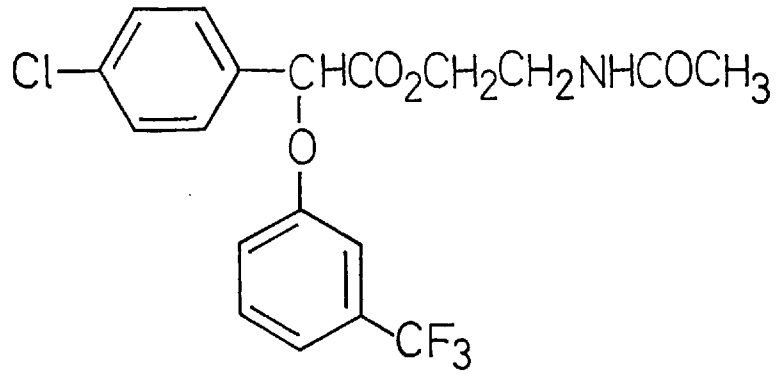
Pyrazinamide Pyrazinoic acid Glycine conjugate

1971). In man and rhesus monkey, 30-40% of the urinary ^{14}C was identified as the ester glucuronide, the remainder being as the unconjugated acid (see Fig.1.10). In rat and dog however, there was extensive hydroxylation of the trifluoromethylphenoxy ring.

A third mode by which an aromatic acid may be synthesized *in vivo* from a xenobiotic which is not an aromatic acid is by the process of aromatization. Unlike hydrolysis and oxidation of the side-chains of aromatic nuclei, aromatization produces the aromatic ring system from an alicyclic system. Lautemann (1863) first observed this phenomenon when he found that (-)-quinic acid (1,3,4,5-tetrahydroxycyclohexanecarboxylic acid), a naturally occurring compound in tea, coffee, fruits and vegetables, was largely excreted as hippuric acid. Aromatization was also observed by Friedmann (1911) who injected dogs with cyclohexanecarboxylic acid and a found a slight increase in hippuric acid excretion. This was confirmed by Bernhard (1937) who proved that aromatization occurred to a greater extent (as much as 59% of dose in one experiment). Later, Adamson *et al.* (1970*a*) showed that (-)-quinic acid was extensively converted to benzoic acid by man and Old World monkeys and demonstrated that in these species the gut flora were responsible for the aromatization (see Fig.1.11). They also found that shikimic acid was aromatized by the gut flora to benzoic acid.

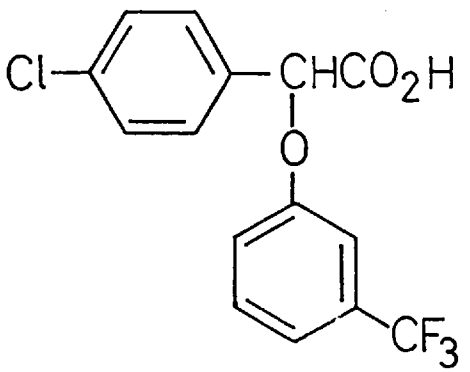
In short therefore, aromatic acids are of considerable importance as foreign componds, and may also arise by Phase I detoxication from a number of metabolic pathways. The factors affecting the extent of their conjugation with various endogenous substances will be discussed.

Fig.1.10 The Biphasic Biotransformation of Halofenate in Man and Rhesus Monkey.



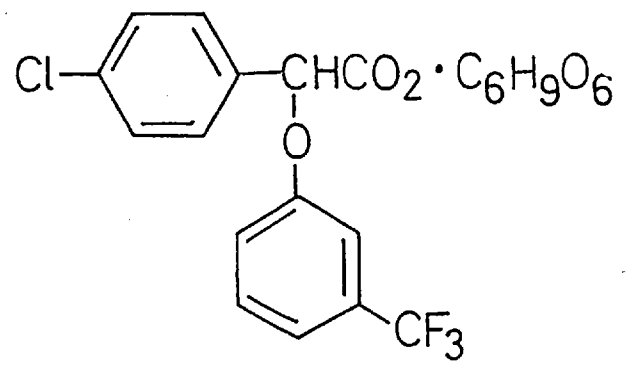
Halofenate

Phase I
hydrolysis



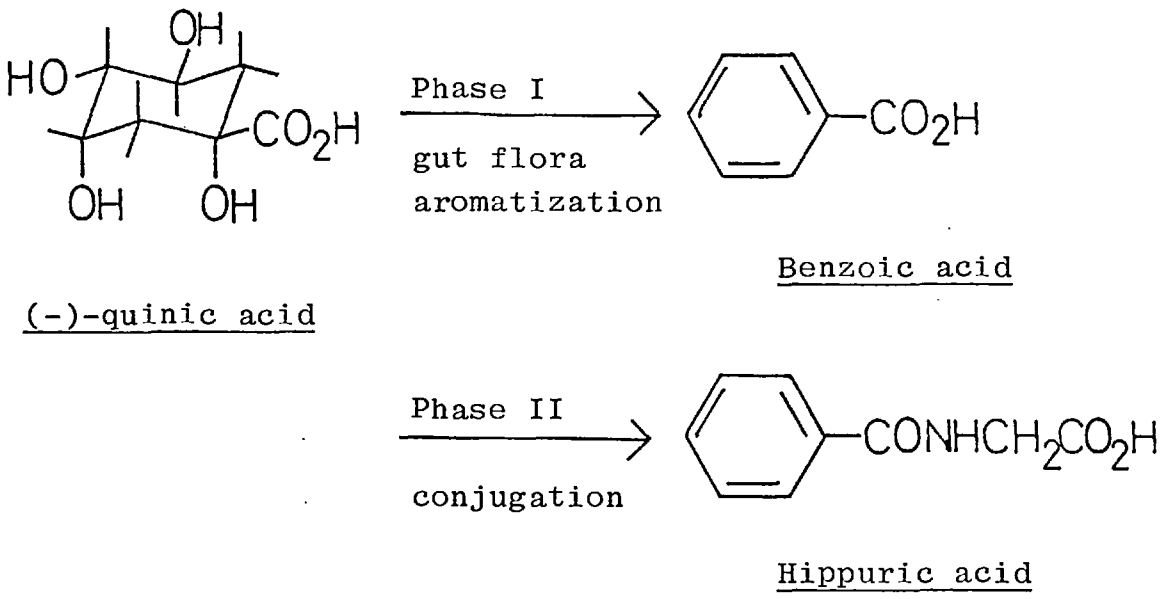
(4-chlorophenyl)(3'-trifluoromethylphenoxy) acetic acid (HFA)

Phase II
conjugation



HFA ester glucuronide

Fig.1.11 The Biphasic Biotransformation of (-)-quinic acid.



(-)-quinic acid

Benzoic acid

Hippuric acid

The degree of conjugation of an aromatic acid with a particular amino acid or with glucuronic acid is undoubtedly related to the physico-chemical properties of that aromatic acid. Such factors might include lipid solubility, acidity, steric and conformational elements. If it is assumed that most species have the ability to carry out many of the known aromatic acid conjugations to a greater or lesser degree, then the extent of conjugation depends on the acid administered. As stated previously, Bridges *et al.* (1970) found that all species studied, except the Indian fruit bat, utilised mainly glycine to conjugate benzoic acid. However, a similar study of the conjugation of phenylacetic acid by James *et al.* (1972*a*) revealed that this compound is only conjugated significantly (>1%) with glycine by prosimian and sub-primate species, the primates utilising glutamine. Taurine was also used to some extent for conjugation by most

species studied. There exists therefore, a dramatic difference in the conjugation of benzoic acid and phenylacetic acid in most species, and this is no more evident than in the Indian fruit bat. This bat does not make hippuric acid from administered benzoic acid (Bridges *et al.*, 1970 ; Bababunmi *et al.*, 1973; Ette *et al.*, 1974) but is capable of making phenaceturic acid (phenylacetylglycine) from phenylacetic acid (Ette *et al.*, 1974). Glycine *N*-acyltransferase activity exists therefore in this species. The conjugation of benzoic acid and phenylacetic acid in man, rhesus monkey, dog, cat, ferret and rat is shown in Table 1.13. Since this thesis is mainly concerned with *in vivo* conjugations in ferret, cat and dog, these species will be contrasted with man and the common laboratory species, rat and rhesus monkey. Inspection of the data reveals that dog, cat and rat metabolise phenylacetic acid in a similar fashion to benzoic acid, whilst man, rhesus monkey and ferret handle both compounds differently. Benzoic acid is therefore conjugated in vertebrates only with glycine and glucuronic acid, except in the hen and certain reptiles, which also use ornithine (Bridges *et al.*, 1970). Phenylacetic acid, it would appear, undergoes a greater diversity of conjugations, being conjugated with glycine, glutamine, taurine, ornithine and glucuronic acid (James *et al.*, 1972*a*). The conjugation of 1-naphthylacetic acid (Dixon *et al.*, 1974) and indol-3-ylacetic acid (Bridges *et al.*, 1974) nas been studied in various species. The metabolism of these acids in man, rhesus monkey, dog, cat and rat is shown in Table 1.14.

From this and similar data, it is difficult to propose which species are the best models for man in the conjugation of aromatic acids. Table 1.15 shows that each of the species

Table 1.13 The Conjugation of Benzoic and Phenylacetic Acids in Certain Species.

(After Bridges *et al.*, 1970; James *et al.*, 1972*a*).

Species	Dose(mg/kg)		% urinary ^{14}C conjugated with									
			unconjugated		glycine		glutamine		taurine		glucuronic acid	
	B	P	B	P	B	P	B	P	B	P	B	P
Man	1	1	0	0	100	tr	0	93	0	6	0	0
Rhesus monkey	20	80	0	55	100	1	0	32	0	14	0	0
Dog	51	80	0	0	82	94	0	0	0	4	18	2
Cat	51	80	tr	1	100	98	0	0	0	1	0	0
Ferret	50	80	9	3	70	43	0	0	0	32	22	22
Rat	50	80	1	0	99	99	0	0	0	1	tr	0

tr = trace

B = benzoic acid; P = phenylacetic acid

Table 1.14 The Conjugation of Indol-3-ylacetic and 1-Naphthylacetic Acids in Certain Species.

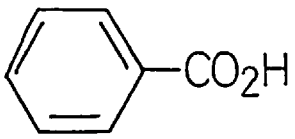
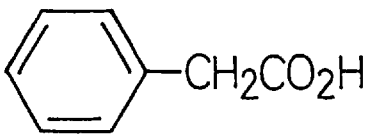
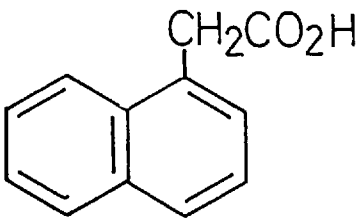
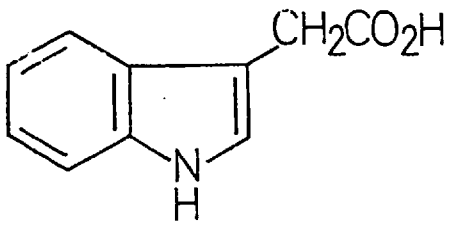
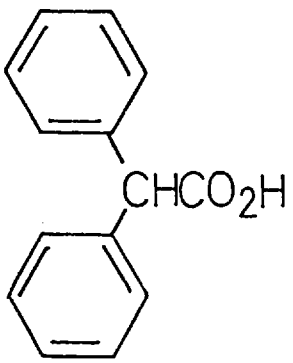
(After Bridges *et al.*, 1974; Dixon *et al.*, 1974).

Species	Dose(mg/kg)		% urinary ^{14}C conjugated with									
			unconjugated		glycine		glutamine		taurine		glucuronic acid	
	I	N	I	N	I	N	I	N	I	N	I	N
Man	∿7	0.125	54	0	0	0	10	0	0	6	35	94
Rhesus monkey	100	100	68	13	0	0	32	0	0	4	0	83
Dog	100	-	30	-	70	-	0	-	0	-	0	-
Cat	100	100	24	2	75	59	0	0	0	39	0	0
Rat	100	100	43	26	57	23	0	0	0	tr	0	51

tr = trace

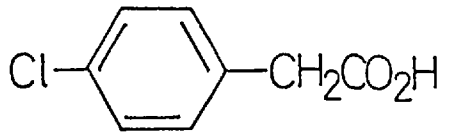
I = indol-3-ylacetic acid; N = 1-naphthylacetic acid

Table 1.15 Species Differences in the Conjugation of Several Aromatic Acids.

		Ref.
 Benzoic acid	GLYCINE (man, rhesus monkey, cat, rat) GLYCINE + GLUCURONIC ACID (dog, ferret)	1
 Phenylacetic acid	GLUTAMINE + TAURINE (man, rhesus monkey) GLYCINE (cat, rat) GLYCINE, TAURINE + GLUCURONIC ACID (dog, ferret)	2
 1-Naphthylacetic acid	GLUCURONIC ACID + TAURINE (man, rhesus monkey) GLUCURONIC ACID + GLYCINE (rat) TAURINE + GLYCINE (cat)	3
 Indol-3-ylacetic acid	GLUCURONIC ACID + GLUTAMINE (man) GLUTAMINE (rhesus monkey) GLYCINE (dog, cat, rat)	4
 Diphenylacetic acid	GLUCURONIC ACID (man, dog, rat)	5,6

/over

Table 1.15 Contd.

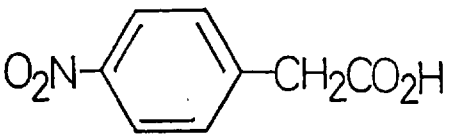


4-Chlorophenylacetic acid

GLUTAMINE (man)

GLUTAMINE + "UNKNOWN" (rhesus monkey) 7

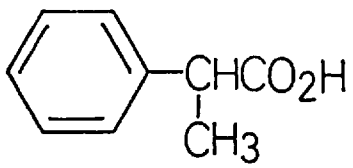
GLYCINE (rat)



4-Nitrophenylacetic acid

"UNKNOWN" (man, rhesus monkey) 7

GLYCINE (rat)



Hydratropic acid

GLUCURONIC ACID (rat) 6

GLYCINE (dog) 8

References

1 Bridges *et al.* (1970)

2 James *et al.* (1972*a*)

3 Dixon *et al.* (1974)

4 Bridges *et al.* (1974)

5 Miriam *et al.* (1927a)

6 Dixon *et al.* (1976)

7 James *et al.* (1972*b*)

8 Kay & Raper (1922)

From the information given by James *et al.* (1972*b*), the "unknowns" were probably the corresponding taurine conjugates.

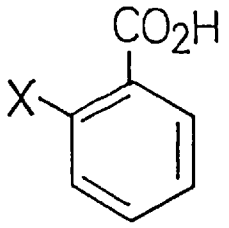
can behave uniquely depending on the structure of the acid administered. Information regarding the major conjugation reactions of aromatic acids, *i.e.* glucuronic acid, glycine, glutamine and taurine conjugation, is extremely fragmentary. Apart from the work cited in Table 1.15, there has been little investigation of species variations of foreign aromatic acid conjugation in mammals. Care must be taken when gathering isolated fragments of this nature and placing an interpretation upon them. Since the pattern of foreign compound metabolism is susceptible both qualitatively and quantitatively to many influences, such as those listed by Williams (1974), differences observed between individual species studied in different laboratories at different times under differing conditions using different methodologies may not be wholly due to true species variations. Because of the difficulty in "controlling out" many such differences in *in vivo* experiments, a certain degree of caution must be excercised when drawing generalisations in this field. Having established that differences in conjugation patterns do exist between species and between structurally similar compounds (*e.g.* benzoic and phenylacetic acids) within individual studies, the various important conjugation reactions of aromatic acids will be discussed individually, with emphasis on the factors affecting their appearance.

Glycine *versus* Glucuronic Acid Conjugation.

The historical aspects of this first known detoxication mechanism are discussed in detail by Williams (1959). Since Keller's clear account of the appearance of hippuric acid in the urine of a dog which had consumed benzoic acid with its

food (Keller, 1842), many other aromatic acids have been shown to be conjugated with glycine in a variety of species. Glycine conjugation is the major route of detoxication for benzoic acid in most species (Bridges *et al.*, 1970). In some species, notably Indian fruit bat, ferret and dog, benzoylglucuronide is also formed. Bray and his colleagues, working with rabbits 20 to 30 years ago, showed that the extent of conjugation of benzoic acids with either glycine or glucuronic acid was influenced by the presence of various substituent groups (see Williams, 1959). When the substituent group, such as 2-, 3- or 4-nitro, conferred a high acidity (pKa 2.2 - 3.4) upon the molecule, the acids were generally excreted unchanged in rabbit urine (Bray *et al.*, 1949). However, pKa had little or no effect upon the ratio of glucuronide and glycine conjugates found in urine (see Table 1.16). Nor, would it appear, does the lipid solubility (log P) of the compound. Table 1.16 shows that the larger the substituent group in the *ortho* position, and thus its steric contribution, the less the conjugation with glycine. This would suggest that penetration of the compound to the site of conjugation, as governed by lipid solubility and degree of ionisation at physiological pH, and thus its distribution in the various body compartments, is not the determining factor in directing the conjugation. Rather, in the case of the 2-substituted benzoic acids studied by Bray, it is a conformational or steric factor (or factors) which governs the extent of glycine conjugation. The degree of steric interaction between *ortho* substituent and carboxyl group is shown in Fig.1.12. The inability of benzoic acids with sterically-demanding *ortho* substituents to be conjugated with glycine

Table 1.16 Factors Affecting the Conjugation of 2-Substituted Benzoic Acids with Glycine in the Rabbit.



X	pKa†	log P§	Approximate diameter of substituent X (nm)×	% dose conjugated with glycine
H	4.2	1.87	0.11	83
F	3.3	-	0.14	43
Cl	2.9	1.98	0.18	5
OH	3.0	2.26	0.17	tr
NH_2	5.0	1.21	0.18	tr
NO_2	2.2	1.52	0.25	0
CH_3	3.9	2.69	0.30	0

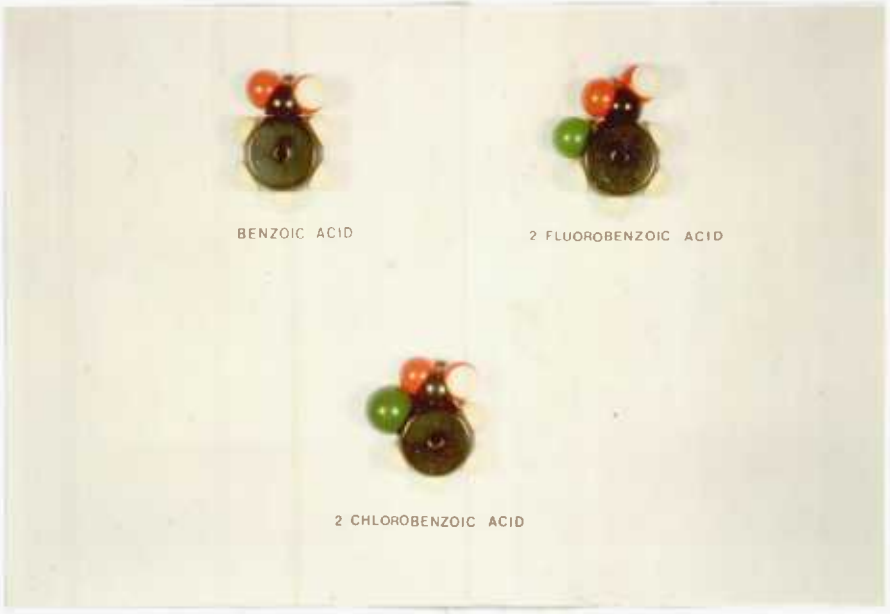
tr means trace

† Figures cited by Williams (1959).

§ Logarithm of partition coefficient between octan-1-ol and pH 7.4 buffer. Values taken from Leo *et al.* (1971).

× Calculated from atomic covalent radii given by Cotton & Wilkinson (1966).

Fig.1.12 Relative Steric Hindrance by *ortho* Substituents.



may be explained in one of three ways:

a) The bulky substituent group hinders the approach of the terminal SH of coenzyme A, thus inhibiting the activation of the acid.

b) The bulky substituent group causes the acyl-CoA to be unstable and to dissociate.

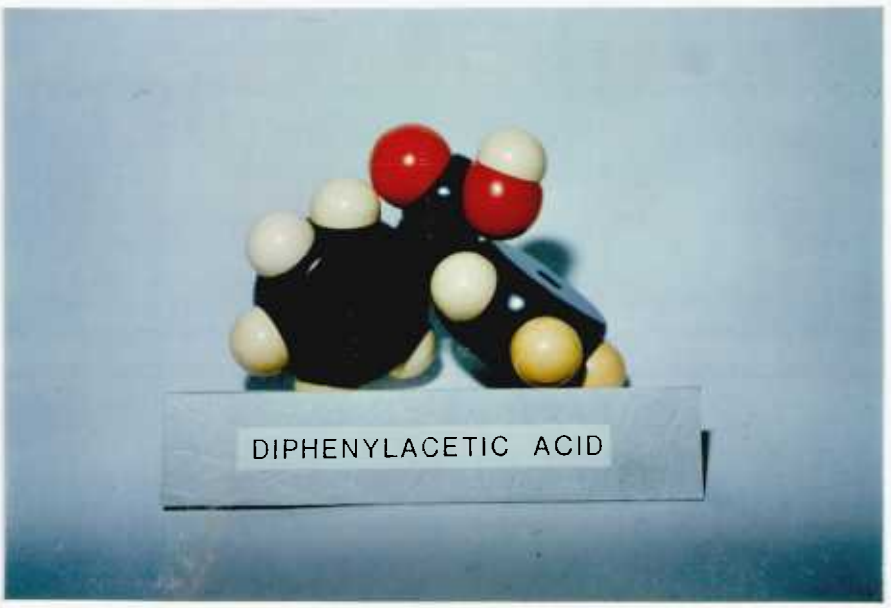
c) Both a) and b).

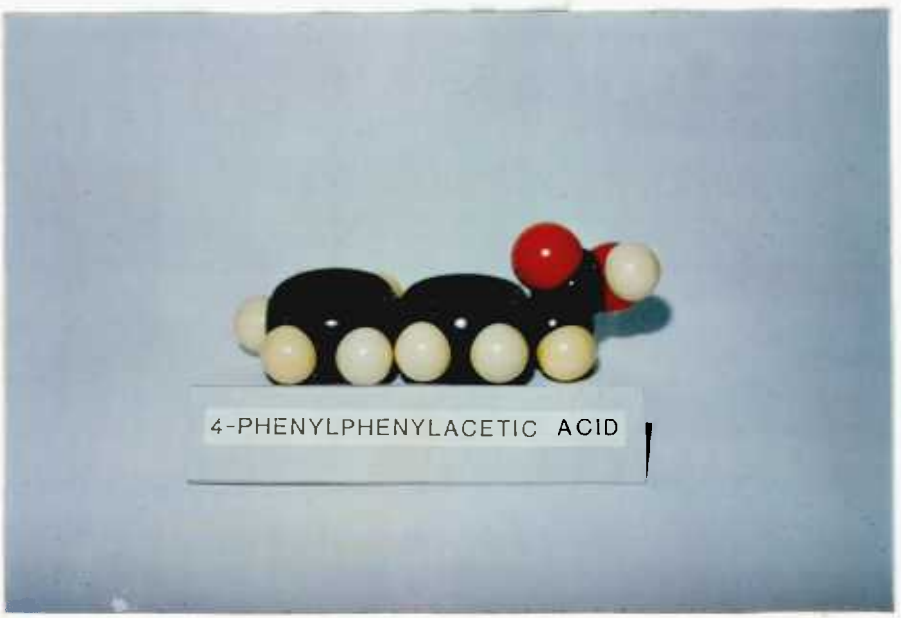
The supposition that steric factors exert their effects on the "activation" step rather than the "transfer" step is consistent with the commonly held view stated previously, that the transferase is amino acid specific but has little donor specificity.

When the body is unable to activate the foreign compound, it must then resort to a conjugation reaction which utilises an activated conjugating agent. Such a reaction is glucuronic acid conjugation.

The pattern of conjugation of many other aromatic acids may similarly be rationalised. Miriam *et al.* (1927a) found that diphenylacetic acid is conjugated with glucuronic acid in the dog, whilst Turnbull *et al.* (1974) found that its structural isomer, 4-phenylphenylacetic acid, is conjugated with taurine but not with glucuronic acid in the dog. Fig.1.13 shows the carboxyl group of diphenylacetic acid to be more sterically hindered than that of 4-phenylphenylacetic acid. Thus, for aromatic acids with longer side-chains than benzoic acid, substitution of bulky groups α to the carboxyl group has a somewhat analogous effect to *ortho* substitution of benzoic acids. Dixon *et al.* (1974) approached the answer when they noticed that the smaller aromatic acids, such as phenylacetic acid and 4-chlorophenylacetic acid, were conjugated with amino

Fig.1.13 Relative Steric Shielding by an α-substituent.

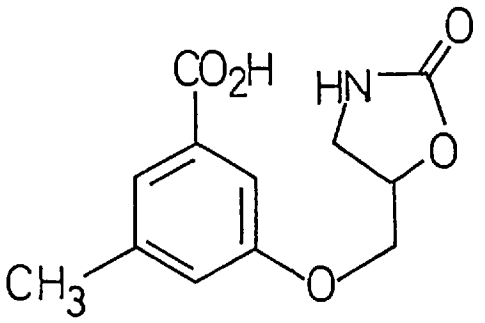




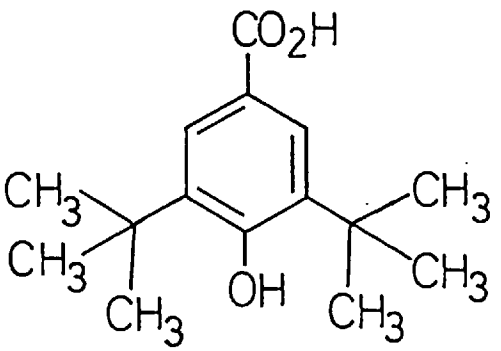
acids, but the more complex (*sic*) carboxylic acids such as diphenylacetic acid, indomethacin, iopanoic acid and iophenoxic acid are conjugated with glucuronic acid. There are many aromatic acids which exemplify this hypothesis of steric control of conjugation. Benzoic acids conjugated with glycine usually have any substituent group remote from the CO_2H group. Such examples are benzoic acid (Bridges *et al.*, 1970), 4-chlorobenzoic acid (McChesney *et al.*, 1967), 4-pyrazinoic acid (Weiner & Tinker, 1972), 4-methoxybenzoic acid (Solheim & Scheline, 1973), 3-trifluoromethylbenzoic acid (Bruce & Maynard, 1968) and 3,4-dichlorobenzoic acid (Knaak & Sullivan, 1968). When the substituent group is large and approaches too close to the CO_2H group, conjugation of the benzoic acid with glucuronic acid occurs. Such examples are 3,5-di-t-butyl-4-hydroxybenzoic acid (Wright *et al.*, 1965), niflumic acid (see Fig.1.5; Lan *et al.*, 1973), 2-aminobenzoic acid (Bray *et al.*, 1948) and 2-methylbenzoic acid (Bray *et al.*, 1949). If the substituent group is large and flexible enough, steric interference may also occur from the *para* position. The uricosuric agent probenecid (see Fig.1.14), which has a *N*-di-n-propylsulphamoyl group *para* to the benzoic acid function, is not conjugated with amino acids but is found in human urine as the ester glucuronide (40% dose, Perel *et al.*, 1970). Likewise the muscle relaxant metaxalone is metabolised to a 3,5-disubstituted benzoic acid which is conjugated with glucuronic acid in dog and man (Bruce *et al.*, 1966). The structure of this metabolite, with its large *meta*-substituent, is shown in Fig. 1.14. Thus, groups of this magnitude in *meta* or *para* positions can influence the conjugation pattern of benzoic acids. For the phenylacetic acids, conjugation with amino acids occurs

Fig.1.14 Steric Hindrance from the *meta* and *para* positions in Benzoic Acids Conjugated only with Glucuronic Acid.

meta interactions

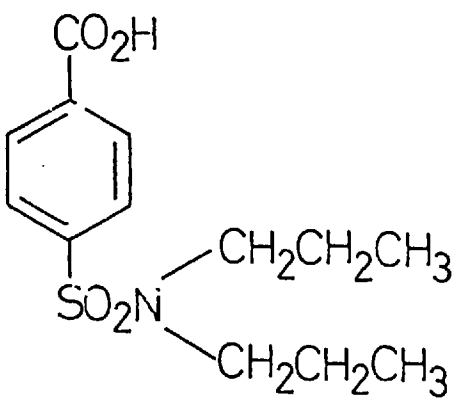


5-(3'-methyl-5'-carboxy phenoxymethyl)-2-oxizolidinone



3,5-di-t-butyl-4-hydroxy benzoic acid

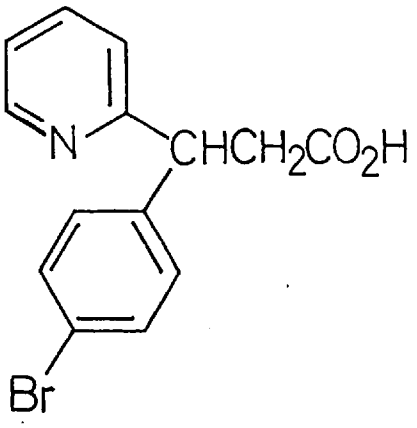
para interactions



4-(*N*-di-n-propylsulphamoyl) benzoic acid (probenecid)

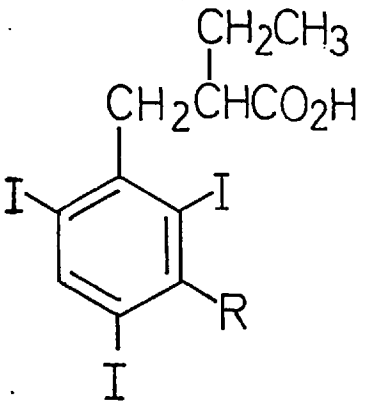
in the absence of bulky substituents, either in the ring or α to the CO_2H group. Such examples are phenylacetic acid (James *et al.*, 1972*a*) and 4-chlorophenylacetic acid (James *et al.*, 1972*b*). However, HFA (see Fig.1.10), indomethacin (see Fig.1.5), diphenylacetic acid and DDA are predominantly conjugated with glucuronic acid. These compounds all possess some degree of steric shielding of the CO_2H group. An interesting test of the hypothesis is the phenylpropionic acids where the CO_2H group should be more susceptible to α substitution effects than to *ortho* substitution effects. The oxidative metabolite of brompheniramine, 3-(4'-bromophenyl)-3-(pyrid-2'-yl)propionic acid, is conjugated with glycine in man and dog (Bruce *et al.*, 1968), whilst the α-ethylphenyl propionic acids, iopanoic acid (McChesney & Hoppe, 1954), tyropanoic acid (McChesney & Hoppe, 1963) and iophenoxic acid (Wade *et al.*, 1971), are conjugated with glucuronic acid in man and dog. The structures of these compounds are given in Fig.1.15. In general, the aryloxy and arylalkyloxyacetic acids are conjugated with amino acids. 4-Chlorophenoxyacetic acid is conjugated with glycine in the rat (Trino *et al.*, 1972) and diphenylmethoxyacetic acid is conjugated with glutamine in the rhesus monkey (Drach & Howell, 1968). 2,4-Dichlorophenoxyacetic acid (2,4-D) has been shown to be excreted quantitatively unchanged by sheep (Clark *et al.*, 1964) and rat (Khanna & Fang, 1966), but recently Grunow & Böhme (1974) demonstrated glycine and taurine conjugation of 2,4-D and 2,4,5-T in rat and mouse. The ether linkage of the phenoxy group allows that group a greater rotational arc than a phenyl group, and thus may be less sterically hindering than a phenyl group. Thus, in fenclofenac (see Fig.1.5), where

Fig.1.15 Structures of some Phenylpropionic Acids and their Conjugation.



man, dog → conjugated with glycine

3-(4'-bromophenyl)-3-(pyrid-2'-yl)propionic acid



man, dog → conjugated with glucuronic acid

R = NH_2, iopanoic acid

R = NHC_4H_9, tyropanoic acid

R = OH, iophenoxic acid

the phenoxy group is *ortho*, there is less influence on the conjugation of the phenylacetic acid than in HFA (see Fig. 1.10), where the phenoxy group is α. Therefore, fenclofenac is able to conjugate with an amino acid, whilst HFA is not.

It must be stressed that the threshold for steric blocking of acyl-CoA formation may vary with species. In general, it may be concluded that the preferential mode of conjugation is with amino acids. This is observed with aromatic acids which are readily activated. In species where the rate-limiting step, which may be determined by rate of mobilisation of glycine (discussed by Williams, 1959) or availability of taurine (discussed later), is the transfer from CoA to amino acid, this may not always be observed. Thus species such as dog and ferret which make appreciable amounts of benzoylglucuronide, do so because of an inherent sluggishness in hippuric acid synthesis. Bridges *et al.* (1970) found that increasing the dose of benzoic acid in the ferret caused an increase in the benzoylglucuronide-hippuric acid ratio. Where glycine conjugation is poor, (dog and ferret), or absent, (Indian fruit bat), for some substrates, then the conjugation is more susceptible to species variations and not just substrate variations. In general, the major factor determining route of conjugation is the structure of the acid, glucuronic acid conjugation occurring with sterically hindered acids, and glycine conjugation occurring with unhindered acids, and an overall species similarity is seen. Species differences can occur in two ways: Firstly when availability of glycine is low and glucuronic acid conjugation takes over. Secondly when the activity of the glycine *N*-acyltransferase is low for various reasons, and other conjugating enzymes come into play, utilising mainly glutamine, taurine or glucuronic acid.

Glutamine Conjugation.

Thierfelder & Sherwin (1914, 1915) found that when man ingested phenylacetic acid it was excreted in the urine as phenacetylglutamine. For many years it seemed that this reaction was restricted to man and to phenylacetic acid, since several species, including the rhesus monkey (Sherwin, 1917), were shown to form the glycine conjugate of phenylacetic acid. However, Power (1936) reported that the chimpanzee also formed phenacetylglutamine. Glutamine conjugates of 3,4-dihydroxy-5-methoxyphenylacetic acid as a metabolite of mescaline (Harley-Mason & Laird, 1959) and 4-methoxyphenylacetic acid and its metabolite 4-hydroxyphenylacetic acid (Oakley & Seakins, 1971) were found in man. Patel & Crawford (1963) showed that four species of African monkey formed a glutamine conjugate with indol-3-ylacetic acid, which was confirmed by Bridges *et al.* (1974) who found indol-3-ylacetylglutamine in six monkey species and man. James *et al.* (1972*a*) demonstrated phenacetylglutamine as a metabolite of phenylacetic acid in twelve species of Old and New World monkeys and in man. From their work, it would appear that glutamine and glycine conjugations are virtually mutually exclusive in man, Old World monkeys and sub-primate species. Only in the New World monkeys were both conjugations found. James *et al.* (1972*b*) showed that man, rhesus monkey and capuchin monkey, but not rat, conjugated 4-chlorophenylacetic acid with glutamine. In the same study, 4-nitrophenylacetic acid was excreted mainly unchanged by man and rhesus monkey. These findings would suggest that glutamine conjugation is restricted to arylacetic acids and occurs only in man and certain monkeys. However, Drach & Howell (1968) found the

glutamine conjugate of diphenylmethoxyacetic acid as a metabolite of diphenhydramine (Benadryl; 2-diphenylmethoxy-*N*,*N*-dimethylethylamine) in the rhesus monkey. Diphenylmethoxyacetic acid is not an arylacetic acid but an arylalkyloxyacetic acid, thus it is evident that glutamine conjugation is specific for the acetic acid part and not the aromatic substituent.

There are a few reports of 4-amino-2-hydroxybenzoic acid (*p*-aminosalicylic acid; PASA) being conjugated with glutamine, but these have never been substantiated by other workers (Smith, 1964). Kawamata & Kashiwagi (1955) claimed that PASA was conjugated with glutamine in man, and Kawamata & Hiratani (1955) published a similar finding for the rat. Hiratani (1957) reported that rat, rabbit and hen formed the glutamine conjugate of PASA. Other Japanese workers of the same period found the glycine conjugate of PASA in man, rabbit, rat and mouse (Nakao *et al.*, 1957). Although these findings do not conform to the general opinion that glutamine conjugation is peculiar to aromatic acetic acids in certain primates, the possibility should not be discounted that glutamine conjugation, like glycine, taurine and glucuronic acid conjugation, is more widely distributed throughout the species, but occurs at a relatively low level in sub-primates.

Taurine Conjugation and Taurine Biochemistry.

Taurine conjugation was originally thought to be unique to the bile acids (see Sobotka, 1937; Haslewood, 1967). Although Kaihara & Price (1961) described a glycyltaurine conjugate of quinaldic acid in the cat, James *et al.* (1971, 1972*a*) were the first to demonstrate conjugation with taurine

alone for xenobiotics. They found that phenylacetic acid was conjugated with taurine to a greater or lesser extent in all species studied except the vampire bat and the domestic hen (James *et al.*, 1972*a*). Phenacetyltaurine was found in significant amounts in the pigeon, ferret, bushbaby, baboon, capuchin monkey, squirrel monkey and mona monkey, but in minor amounts in other species. In a subsequent paper (James *et al.*, 1972*b*), they reported polar unidentified metabolites of both 4-chloro- and 4-nitrophenylacetic acids, which were conjugates of these acids. From the evidence they gave, it seemed likely that these were both taurine conjugates. The anti-inflammatory agent fenclofenac (see Fig.1.5) is found in urine conjugated almost exclusively with taurine in the dog after oral administration (10mg/kg). Glycine conjugation was virtually undetectable (Jordan & Rance, 1974). Another anti-inflammatory agent 4-(cyclopropylcarbonyl)phenylacetic acid (SQ 20, 650) and its reduced metabolite 4-(α-cyclopropyl-α-hydroxymethyl)phenylacetic acid are both conjuagated with taurine in the dog (Lan *et al.*, 1975). Turnbull *et al.* (1974) showed that 4-phenylphenylacetic acid, an oxidative metabolite of the anti-inflammatory agent 4-methyl-2-(4'-phenylbenzyl)-2-oxazoline-4-methanol (AH-5318), is excreted in dog urine as the taurine conjugate (68% urinary ^{14}C calculated as AH-5318). Grunow & Böhme (1974) showed the taurine conjugates of 2,4-D and 2,4,5-T in rat and mouse. To date, these are the only published compounds conjugated with taurine.

Thus it would appear that, like glutamine conjugation, taurine conjugation occurs only with arylacetic and aryloxy-acetic acids, *i.e.* aromatic acetic acids.

There is no information in the literature regarding the

mechanism of taurine conjugation of xenobiotic acids. For the present it must be assumed that this reaction proceeds like glycine conjugation, with activation to a CoA intermediate and transfer utilising a taurine *N*-acyltransferase. This is indeed analagous also to the system conjugating the bile acids with taurine (Scherstén, 1970).

The biochemistry and physiology of taurine has been extensively reviewed (Jacobsen & Smith, 1968). Taurine was first reported as a constituent of living tissue in ox bile by Tiedemann & Gmelin (1827), who called it "bile-asparagine", and it is now known to be one of the most abundant amino acid analogues in the body. However, only one physiological function for taurine is properly established; that of bile acid conjugation (Sturman *et al.*, 1975). Taurine is ubiquitous in the body, but its quantitative distribution is known to vary with species. Table 1.17 gives tissue concentrations of free taurine in several species. Where possible, values for the main drug metabolising organs, liver and kidney, have been quoted. Values for spleen have been included for comparison. The two species with considerable hepatic taurine levels are dog (7.2µmol/g wet weight) and cat (8.3µmol/g wet weight). Rabbit, cat and guinea pig levels are relatively low (0 - 0.5, 0.6 - 4 and 0 respectively). It is possible that for acids such as phenylacetic acid, which are conjugated mainly with amino acids in most species, and are thus readily converted into a CoA intermediate, the availability of taurine determines the extent of reaction between acyl-CoA and taurine. This is consistent with the observation of taurine conjugation in the dog for many acids, but not in other species such as the rat, guinea pig and rabbit.

Table 1.17 Taurine Levels in Some Mammalian Tissues.

Species	Tissue	Taurine concentration (μmol/g wet weight)	Reference
Man	Liver	0.3 - 1.8	1
	Kidney	1.4 - 1.8	1
	Spleen	11.4	1
Rabbit	Liver	0.0 - 0.5	2
	Kidney	1.9 - 3.8	2
	Spleen	5.2 - 5.8	3
Mouse	Spleen	11 - 12	3
Rat	Liver	0.6 - 4.0	4
	Kidney	5 - 11.8	4
	Spleen	7 - 14.7	4
Guinea pig	Liver	0	5
	Kidney	1.0 - 1.8	5
	Spleen	5.2 - 5.6	5
Dog	Liver	7.2	6
Cat	Liver	8.3	7
	Kidney	3.1	7

References

1 Zachmann *et al.* (1966)

2 Dubreuil & Timiras (1953)

3 Kit & Awapara (1953)

4 Garvin (1960)

5 Awapara (1956)

6 Jacobsen & Smith (1963)

7 Tallan *et al.* (1954)

The Use of Less Common Species in Foreign Compound Metabolism Studies.

Of the 4000 or so species of mammals, very few have been studied from a comparative toxicological or biochemical standpoint. As underlined by Smith (1974), knowledge in this respect is largely confined to a few rodent species (rat, mouse and guinea pig), a few carnivores (dog, cat and ferret, although the latter has not been rigorously investigated), a lagomorph (the rabbit) and a few simian species (squirrel monkey, rhesus monkey and marmoset). The response of the animal to an external stimulus may often be species-dependent, and this may raise various problems in data interpretation. To obtain a better picture of the overall effect of foreign compounds upon the environment, work must not be focused too closely on a few restricted species, but rather extended more uniformly throughout the phyla.

It has been proposed that the rhesus monkey (*Macaca mulatta*) provides the best model at present for man in studies of the disposition and metabolism of foreign compounds (Smith & Caldwell, 1976). The search for cheaper or more convenient species to perform this task must continue, and this is indeed advocated by the World Health Organisation (1975) and by the Committee on Problems of Drug Safety (1969).

A study of the biochemistry of less common species is of interest *per se*. The phylogenetic development of enzymes and other proteins, such as haemoglobins, has been investigated in the context of species classification, *i.e.* taxonomy (Watts, 1968). One of the future developments in foreign compound metabolism studies may be to assist in the proper classification of species. For this purpose, Williams

(1976) has derived the term pharmacozootaxonomy. French *et al.* (1974) have shown the lion (*Panthera leo*), civet (*Viverra civetta*) and genet (*Genetta pardina*) to have a defect in glucuronic acid conjugation similar to the domestic cat (*Felis catus*). Thus a pharmacozootaxic classification may be achieved which, in this case, is consistent with anatomical and morphological taxonomy, that all 4 species belong to the superfamily Feloidea. Similarly, Bridges & Williams (1963) showed that the fox was similar to the dog in its apparent inability to acetylate aromatic amino groups. Caldwell *et al.* (1975) showed that the hyaena (*Crocuta crocuta*) is like the cat with respect to N^4-acetylation of sulphadimethoxine. The hyaena is nominally classified in the cat superfamily (Ewer, 1973), but future pharmacozootaxic studies may suggest that it requires a classification intermediate between the Canoidea and Feloidea.

Two of the species used in this study, the ferret and the bat, are not normally encountered in the laboratory, although the former appears to be of increasing popularity. Using the ferret, other workers have studied the *in vivo* metabolism of benzoic acid (Bridges *et al.*, 1970), phenylacetic acid (James *et al.*, 1972), quinic acid (Adamson *et al.*, 1970*a*), sulphadimethoxine (Adamson *et al.*, 1970*b*), phenol (Capel *et al.*, 1972), chlorobenzene (French, 1970) and coumarin (Mead *et al.*, 1958). In a review of the biomedical use of ferrets in research, Hahn & Wester (1969) give little information on the drug-metabolising prowess of the ferret, and it appears that the main laboratory uses of ferrets lie in the fields of virology and immunology. Liver preparations from ferrets have been found to catalyse the conjugation of

some epoxides with glutathionine (Boyland & Williams, 1965) and the activity of the ferret glutathionine *S*-transferases is given by Chasseaud (1973). Ioannides & Parke (1975) have studied some aspects of the perinatal pharmacology of the ferret, and have found that the hepatic microsomal drug-metabolising enzymes exhibit very low activity at birth and increase rapidly with age and are still increasing at 8 weeks.

Although the bat is the second largest Order of mammals after the rodent (Smith, 1974), little work in biochemical, pharmacological or toxicological areas have been performed in any of the 981 known species of bat. A few biochemical parameters have been determined, such as blood sugar (Dodgen & Blood, 1956), glycogen (Troyer, 1959) and phosphorylase (Leonard & Wimsatt, 1959). The microsomal drug-metabolising ability of two species of eastern American bats (*Myotis lucifugus* and *Plecotus rafinesquii*) has been investigated (Litterst *et al.*, 1974), mainly instigated by the observation of Luckens & Davis (1964) that bats were unusually sensitive to DDT poisoning. The former found that although the microsomal enzyme activities were qualitatively similar to other groups of mammals, ethylmorphine *N*-demethylase and NADPH cytochrome c reductase activities were only about 20-25% of those found for mouse, guinea pig and rat. The metabolism of three xenobiotics in the intact bat has been published. These are phenylacetic acid in the vampire bat (*Desmodus rotundus*; James *et al.*, 1972) and Indian fruit bat (*Pteropus giganteus*; Ette *et al.*, 1974), benzoic acid in the Indian fruit bat (Bridges *et al.*, 1970; Bababunmi *et al.*, 1973; Ette *et al.*, 1974) and 1-naphthylacetic acid in the Indian fruit bat (Dixon *et al.*, 1974). This work suggests that the Indian fruit bat

does not make hippuric acid or 1-naphthylacetylglycine but is able to make phenylacetylglycine. Obviously more work is required to determine whether other species of bat are unable to synthesize hippuric acid, and whether the Indian fruit bat is unable to synthesize the glycine conjugates of substituted benzoic acids.

Aim of the Present Study.

The aim of this work is to determine the extent of conjugation with taurine *in vivo* of a series of aromatic acids in the ferret (*Mustela furo*), dog (*Canis familiaris*) and cat (*Felis catus*). Within the scope of this work falls a clearer definition of the factors giving rise to taurine conjugation of foreign aromatic acids, such as structure of the acid, dose and species.

CHAPTER TWO

Materials and Methods

Contents

List of Tables

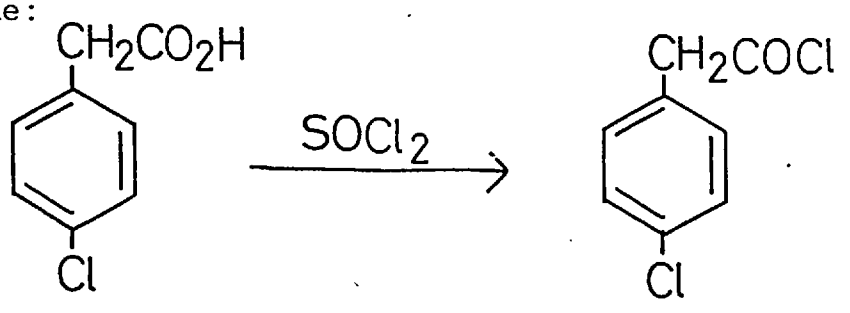
Synthesis and Characterization of a Series of *N*-acyltaurines (Taurine Conjugates).

Information in the chemical literature regarding the synthesis of *N*-substituted taurine derivatives is very sparse. Gandini (1946) described the synthesis of a series of alicyclic taurines, whilst Fieser *et al.* (1956) and Seidenfaden (1961) have synthesized some long-chain acyltaurines for use as emulsifying agents and cosmetic additives. These methods all employ the basic Schotten-Baumann reaction of an acid chloride with an amine, and it is this method that was used here. A laborious method for the synthesis of benzoyltaurine is also described by Josephson (1933).

The isolation of taurine conjugates, and sulphonic acids in general, poses a major problem due to their poor extractability from aqueous media. Sulphonic acids may have pKa values of the order -0.3 to 2.5, and are thus very strong acids indeed. [Kortüm *et al.* (1961) give pKa values of -0.33, 0.17 and 2.55 for 1-aminoethylsulphonic acid, 1-naphthylsulphonic acid and phenylsulphonic acid respectively]. A simple method for the synthesis and isolation of taurine conjugation, without recourse to solvent extraction, is described:

1. 4-Chlorophenacetyltaurine.

The compound was prepared according to the following scheme:



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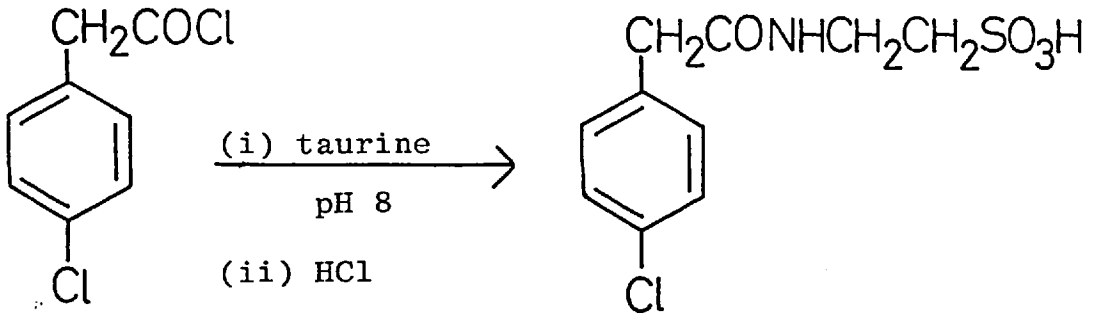


Thionyl chloride (8.2g; 70mmol) in benzene (25ml) was added dropwise to a refluxing solution of 4-chlorophenylacetic acid (10.0g; 59mmol) in benzene (150ml). The mixture was refluxed for 3h and allowed to stand overnight. Excess thionyl chloride and benzene were removed in a rotary evaporator and the oily residue distilled *in vacuo* to give a yellow oil b.p.(2mm) = 120-140^{0}C; YIELD = 2.78g (25%).

4-Chlorophenylacetyl chloride had a carbonyl stretching frequency, υ(C=O) = 1810cm^{-1}.

4-chlorophenylacetyl chloride (1.0g; 5.3mmol) was added dropwise with stirring to a cooled (0-5^{0}C) solution of taurine (0.625g; 5.0mmol) in water (20ml) at pH8. The solution was stirred at 0-5^{0}C for 2h, allowed to warm to room temperature over 3h, and acidified to pH1 with 11.6M-HCl.

The solution was extracted with diethyl ether (3x1vol) to remove excess acid chloride and any free acid. The aqueous phase was reduced to dryness in a rotary evaporator (<40^{0}C) and the solid residue triturated with hot methanol (50ml). The methanol solution was reduced to about 5ml in a rotary evaporator and allowed to crystallise. *4-Chlorophenylacetyl-taurine* was recrystallised from a large volume of boiling methanol, giving small white crystals m.p. 234-235^{0}C; YIELD = 0.2g (15%). (Found: C, 43.1; H, 4.35; N, 4.83%.

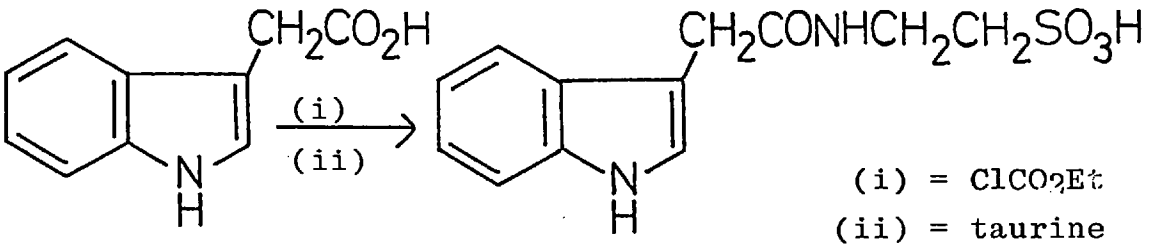
$C_{10}H_{12}ClNO_4S$ requires C, 43.3; H, 4.36; N, 5.04%).

The product was further investigated using infra-red spectroscopy and mass spectrometry. The essentials of the infra-red spectrum are given in Appendix 1 and the mass spectrum, together with the proposed fragmentation pattern of the methyl ester, are shown in Appendix 2.

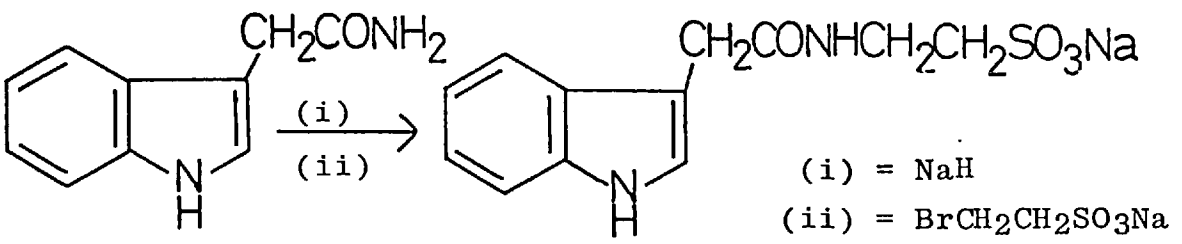
2. Indol-3-ylacetyltaurine.

Several attempts to prepare indol-3-ylacetyl chloride failed. There is little information regarding this compound in the literature. Indol-3-ylacetyltaurine was eventually prepared by the following two schemes:

a)



b)



a) "The mixed anhydride route".

Indolylacetic acid (3.12g) and tri-n-butylamine (3.72g) were dissolved in dioxan (100ml) at 10°C and then ethyl chloroformate (2ml) was added with stirring. After keeping the mixture at 10°C for 30min, a solution of taurine (1.37g) in 1M-NaOH (11ml) was added and the mixture allowed to stand

for 30min. The whole was then evaporated to dryness at 35°C in a rotary evaporator. The residue was then extracted with a 2:1(v/v) chloroform-methanol mixture (100ml) and filtered to remove taurine and NaCl. The filtrate was taken to near dryness at 35°C and the residue dissolved in 95% ethanol (20ml). This solution was passed slowly through a cation exchange column (Dowex 50W-X8: 20-50 U.S. Mesh(H), prepared in 95% ethanol) to remove tributylamine. The eluate (20ml) was treated with sufficient sodium ethoxide in ethanol to change the colour from red to yellow. The mixture was filtered and the filtrate reduced to dryness at 35°C. The residue was dissolved in water (20ml), extracted with ether 2x20ml) and the aqueous layer freeze-dried. The residue was extracted with boiling ethanol (20ml) and on cooling the extract yellowish crystals separated. These were recrystallised from 95% ethanol to give the crude sodium salt of *indol-3-ylacetyltaurine* (1.8g), m.p. 213°C. This was probably a mixture of the sodium salt and the free acid and could not be obtained analytically pure. On chromatography on Whatman No.1 paper in butan-1-ol-acetic acid-water (12:3:5v/v) it ran as a single spot of R_F 0.45. The compound (5mg) was heated on a water bath for 6h with 6M-HCl (5ml) and a portion (10µl) of the solution was chromatographed as before. On spraying the chromatogram with 2% 4-dimethylaminobenzaldehyde in 2M-HCl (Ehrlich's reagent), a blue spot of R_F 0.9 corresponding to indolylacetic acid was obtained, and on spraying a similar chromatogram with 0.1% ninhydrin in acetone, a single spot of R_F 0.1 corresponding to taurine was found.

b) Via Indol-3-ylacetamide.

Indol-3-ylacetamide (1.74g; 0.01 mol) in dry dimethyl-

formamide (DMF; 20ml) was added dropwise to a refluxing suspension of sodium hydride (0.24g; 0.01mol) in dry DMF (20ml). The resulting solution turned a deep purple colour and an increase in reflux rate was observed indicating the evolution of hydrogen and the formation of the anion. After 30min, 2-bromoethane sulphonic acid sodium salt (2.3g; 0.11mol) in hot (100°C) dry DMF (50ml) was added. The mixture was then refluxed for 2h and allowed to stand overnight. The solution was poured into water (500ml) and extracted with chloroform (3x200ml). The aqueous phase was reduced to dryness in a rotary evaporator and a portion (1mg) of the resulting brown solid examined by thin-layer chromatography (t.l.c.) on pre-coated aluminium-backed t.l.c. plates (silica gel $60F_{254}$, 0.2mm thickness; Merck, Darmstadt, W. Germany) in chloroform-methanol (4:1v/v). Two spots were found positive to Ehrlich's reagent, the least polar (R_F 0.50) corresponding to indol-3-ylacetamide and the more polar (R_F 0.10) corresponding to indol-3-ylacetyltaurine prepared by the "mixed anhydride route". The brown residue was then extracted with boiling acetone, leaving a yellowish powder, m.p. >300°C; YIELD = 0.35g (13%).

This substance was examined further by infra-red spectroscopy and mass spectrometry and was found to have an identical mass spectrum to that obtained with the substance prepared by route a). The infra-red spectrum and mass spectrum are given in Appendices 1 and 2 respectively.

3. General Method for the Preparation of Taurine Conjugates.

Eight other taurine conjugates were prepared using the following scheme:

$$RCOCl \xrightarrow[pH\ 8]{H_2NCH_2CH_2SO_3H} RCONHCH_2CH_2SO_3Na$$

Acyl chloride (0.11mol) was added dropwise to a stirred and cooled (0-5°C) solution of taurine (12.5g; 0.10mol) in 1M-NaOH (110ml; 0.11mol). The solution was stirred for 2h at 0-5°C and then allowed to warm to room temperature and stir overnight. The solution was extracted with diethyl ether (3x200ml) to remove acyl chloride and carboxylic acid, and the aqueous layer reduced to dryness at 35°C in a rotary evaporator. The taurine conjugate was extracted from taurine and NaCl with boiling MeOH (500ml), the methanol solution filtered and reduced to a small volume (50ml) in a rotary evaporator. The addition of approximately 5 volumes of acetone precipitated the conjugate, which was filtered, triturated with diethyl ether, dried and recrystallised from methanol-acetone mixtures. The melting points and yields are given in Table 2.1. Due to the high acidity of taurine derivatives, they were usually isolated as a mixture of the sodium salt and the free acid. All the synthesized taurine derivatives were examined by infra-red spectroscopy and the results are shown in Appendix 1. The mass spectra of certain of the taurine conjugates are given in Appendix 2.

Compounds and Radiochemicals.

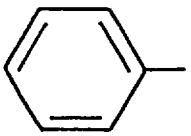
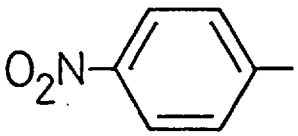
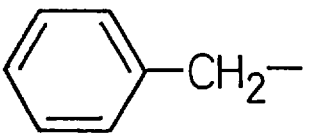
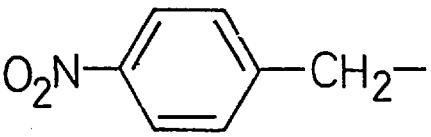
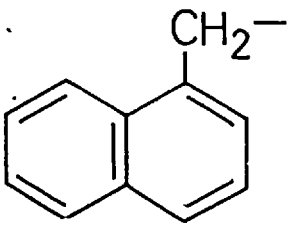
(i) Phenylacetic acid

Phenyl[*carboxy*-^{14}C]acetic acid (specific activity 59.0 mCi/mmol) was obtained from the Radiochemical Centre,

Table 2.1 Melting Points and Yields of some *N*-acyltaurines.

Melting points (capillary tube) are uncorrected.

$$RCONHCH_2CH_2SO_3H$$

R	m.p.(°C)	Yield (%)
	295-300	20
	278-280	61
	225-227	61
	183-185	65
	229-231	34
CH_3-	>300(d)	25
CH_3CH_2-	>300(d)	10
$CH_3CH_2CH_2-$	>300(d)	10

Amersham. Phenylacetic acid (m.p. 77-78°C) was purchased from Hopkin and Williams, London. The melting points and sources of the other phenylacetic acid derivatives used are shown in Table 2.2, and the R_F values of some possible metabolites of phenylacetic acid are given in Table 2.3.

(ii) 4-Chlorophenylacetic acid.

4-Chlorophenyl[*carboxy*-^{14}C]acetic acid (specific activity 0.183 mCi/mmol) was available within the department. 4-Chlorophenylacetic acid (m.p. 103-105°C) was purchased from the Aldrich Chemical Company Inc., U.K. 4-Chlorophenylacetylglycine (m.p. 157-159°C) and 4-chlorophenylacetyl-L-(+)-glutamine (m.p. 147°C) were available within the department. The synthesis of 4-chlorophenylacetyltaurine has been described. The R_F values of 4-chlorophenylacetic acid and some possible metabolites are given in Tables 2.4 and 2.5.

(iii) 4-Nitrophenylacetic acid.

4-Nitrophenyl[*carboxy*-^{14}C]acetic acid (specific activity 0.76 mCi/mmol) was available in the department. 4-Nitrophenylacetic acid (m.p. 154-155°C) was purchased from Koch-Light, Colnbrook, U.K. 4-Nitrophenylacetylglycine (m.p. 157-159°C), 4-nitrophenylacetyl-L-(+)-glutamine (m.p. 185-186°C), 4-aminophenylacetic acid (m.p. 201°C (d)) and 4-acetylaminophenylacetic acid (m.p. 160°C) were available within the department. The synthesis of 4-nitrophenylacetyltaurine has been described. The R_F values of 4-nitrophenylacetic acid and some possible metabolites are given in Table 2.6.

(iv) Benzoic acid.

[*Carboxy*-^{14}C]benzoic acid (specific activity 49.9 mCi/mmol)

Table 2.2 Phenylacetic Acid Derivatives.

Compound	m.p.(°C)	Source
2-Hydroxyphenylacetic acid	147 - 149	A
3-Hydroxyphenylacetic acid	131 - 134	A
4-Hydroxyphenylacetic acid	149 - 151	K
3,4-Dihydroxyphenylacetic acid	127 - 130	A
(±)-Mandelic acid	118 - 120	K
Phenylacetylglycine	143	D
Phenylacetyl-L-(+)-glutamine	101	P
Phenylacetyltaurine	225 - 227	P
Phenylacetyl-L-(+)-alanine	149 - 150	P
N^1,N^5-Diphenylacetyl-L-(+)-ornithine	140	D
Phenylacetyl-L-(+)-asparagine	183 - 184	D
Phenylacetylglycylglycine	170 - 171	P
Phenylacetyl-L-(+)-glutamic acid	118	D
Phenylacetyl-L-(+)-threonine	152 - 153	D

A = Aldrich Chemical Company Inc., U.K.

K = Koch-Light, Colnbrook, Bucks, U.K.

P = Prepared by the Schotten-Baumann method.

D = Available within this department.

Table 2.3 Thin-layer Chromatography of Phenylacetic Acid and some Possible Metabolites.

Silica gel 60 F_{254} plates (0.2mm thickness) were used (Merck, Darmstadt, W.Germany).

Compounds were located as quenched spots under U.V. light (Hanovia Chromatolite Lamp).

Compound	R_F in system		
	A	B	C
Phenylacetic acid	0.80	0.70	0.90
2-Hydroxyphenylacetic acid	-	0.55	-
3-Hydroxyphenylacetic acid	-	0.51	-
4-Hydroxyphenylacetic acid	0.76	0.52	0.87
3,4-Dihydroxyphenylacetic acid	-	0.33	-
(±)-Mandelic acid	-	0.27	-
Phenylacetylglycine	0.56	0.24	0.60
Phenylacetyl-L-(+)-glutamine	0.39	0.09	0.32
Phenylacetyltaurine	0.16	0.01	0.07
Phenylacetyl-L-(+)-alanine	0.66	0.34	0.75
N^1,N^5-Diphenylacetyl-L-(+)-ornithine	0.60	0.20	0.56
Phenylacetyl-L-(+)-asparagine	0.39	-	0.25
Phenylacetylglycylglycine	0.33	-	0.29
Phenylacetyl-L-(+)-glutamic acid	0.55	-	0.60
Phenylacetyl-L-(+)-threonine	0.53	-	0.52

A = Benzene-acetone-glacial acetic acid (2:2:1 v/v)

B = Benzene-acetone-glacial acetic acid (6:2:1 v/v)

C = Acetone-benzene-pentan-1-ol-glacial acetic acid (5:4:2:1 v/v)

Table 2.4 Paper Chromatography of 4-Chlorophenylacetic Acid and some possible metabolites.

Whatman 3mm paper with the descending method was used. The solvent systems were: D, chloroform : methanol (4:1 v/v); E, butan-2-one : 1% aqueous sodium acetate : glacial acetic acid (10:5:2 v/v); F, butan-1-ol : glacial acetic acid : water (4:1:2 v/v). The colour test used was a modification of the chlorine-starch/KI reagent described by James *et al.* (1972*a*). The chromatograms were sprayed with 5% aqueous sodium hypochlorite and dried in an oven at 100°C over a beaker containing a small quantity of glacial acetic acid. This generated chlorine *in situ*. The chromatograms were then sprayed with starch-KI solution. The amino acid conjugates appeared as a brown spot on a white background.

Compound	*RF* values in system			Colour Reaction
	D	E	F	
4-Chlorophenylacetic acid	0.97	0.97	0.95	none
4-Chlorophenylacetyl-glycine	0.10	0.93	0.91	brown spot
4-Chlorophenylacetyl-taurine	0.10	0.67	0.58	brown spot

Table 2.5 Thin-Layer Chromatography of 4-Chlorophenylacetic Acid and some Possible Metabolites.

Method as described in Table 2.3.

Compound	R_F in system	
	A	C
4-Chlorophenylacetic acid	0.84	0.93
4-Chlorophenylacetylglycine	0.62	0.61
4-Chlorophenylacetyl-L-(+)-glutamine	0.43	0.34
4-Chlorophenylacetyltaurine	0.16	0.11

Table 2.6 Thin-Layer Chromatography of 4-Nitrophenylacetic Acid and some Possible Metabolites.

Method as described in Table 2.3.

Compound	R_F in system	
	C	G
4-Nitrophenylacetic acid	0.87	0.87
4-Nitrophenylacetylglycine	0.49	0.67
4-Nitrophenylacetyl-L-(+)-glutamine	0.23	-
4-Nitrophenylacetyltaurine	0.10	0.29
4-Aminophenylacetic acid	0.64	0.43
4-Acetylaminophenylacetic acid	0.75	0.76

A = Benzene-acetone-glacial acetic acid (2:2:1 v/v)

C = Acetone-benzene-pentan-1-ol-glacial acetic acid (5:4:2:1 v/v)

G = Benzene-acetone-pentan-1-ol-glacial acetic acid (1:1:1:1 v/v)

was obtained from the Radiochemical Centre, Amersham, U.K. Benzoic acid (m.p. 122°C) was purchased from Hopkin and Williams, U.K. Cyclohexanecarboxylic acid (m.p. 30-32°C, b.p. 232-233°C) was obtained from the Aldrich Chemical Company Inc., U.K. Benzoylglutamic acid (m.p. 152-153°C; Nutritional Biochemicals Corporation, Cleveland, Ohio), benzoyl-α-alanine, benzoyl-β-alanine and benzoylvaline (Sigma Chemical Company Ltd., Kingston-upon-Thames, U.K.) were purchased. 4-Hydroxybenzoic acid (m.p. 214-215°C), hippuric acid (m.p. 188-191°C), 4-hydroxyhippuric acid (m.p. 238°C), benzoylglucuronide (m.p. 187°C) and N^1,N^5-dibenzoyl-ornithine (m.p. 181.5-182°C) were all available in the department. The synthesis of benzoyltaurine has been described. The R_F values of benzoic acid and some possible metabolites are given in Table 2.7.

(v) 4-Nitrobenzoic acid.

4-Nitro[*carboxy*-^{14}C]benzoic acid (specific activity 6.0 mCi/mmol) was purhased from ICN, Irvine, California. 4-Nitrobenzoic acid (m.p. 239-241°C) and 4-aminobenzoic acid (m.p. 188-189°C) were purchased from Hopkin and Williams, U.K. 4-Nitrohippuric acid (m.p. 128-131°C; Aldrich Chemical Company Inc., U.K.), 4-aminohippuric acid (m.p. 199-200°C (d); Koch-Light, Colnbrook, U.K.), 4-nitrobenzoylglutamic acid (m.p.105-108°C; Nutritional Biochemicals Corporation, Cleveland, Ohio) and 4-aminobenzoylglutamic acid (m.p. 173°C; Nutritional Biochemicals Corporation, Cleveland, Ohio) were all purchased. 4-Acetylaminobenzoic acid (m.p. 259-262°C (d)) and 4-acetylaminohippuric acid (m.p. 228-230°C) were available in the department. The synthesis of 4-nitrobenzoyltaurine has been described. R_F values of 4-nitrobenzoic acid and some possible

metabolites are given in Table.2.8.

Table 2.7 Thin-Layer Chromatography of Benzoic Acid and some Possible Metabolites.

Method as described in Table 2.3.

Compound	R_F in system		
	A	B	C
Cyclohexanecarboxylic acid*	0.83	0.65	-
Benzoic acid	0.79	0.60	0.97
4-Hydroxybenzoic acid	0.75	0.53	0.93
Benzoyl-D,L-valine	0.71	0.47	0.87
Benzoyl-β-alanine	0.63	0.36	0.82
Benzoyl-D,L-α-alanine	0.63	0.35	0.77
N^1,N^5-dibenzoyl - L-(+)-ornithine	0.57	0.23	0.69
Hippuric acid	0.51	0.23	0.61
Benzoyl-L-(+)-glutamic acid	0.51	0.18	0.57
4-Hydroxyhippuric acid	0.46	0.11	-
Benzoyl-β-D-glucuronide	0.16	0.01	0.10
Benzoyltaurine	0.13	0.01	0.07
Benzoyl-L-(+)-arginine	0.05	0.00	0.01

A = Benzene-acetone-glacial acetic acid (2:2:1 v/v)

B = Benzene-acetone-glacial acetic acid (6:2:1 v/v)

C = Acetone-benzene-pentan-1-ol-glacial acetic acid (5:4:2:1 v/v)

* Located with chromosulphuric acid spray (Bertetti, 1954).

Table 2.8 Thin-Layer Chromatography of 4-Nitrobenzoic Acid and some Possible Metabolites.

Method as described in Table 2.3.

Chromatograms were sprayed with Ehrlich's reagent.

Compound	R_F in system		Colour reaction
	A	G	
4-Nitrobenzoic acid	0.77	0.82	none
4-Nitrohippuric acid	0.52	0.63	purple
4-Nitrobenzoyltaurine	0.16	0.28	pale yellow
4-Nitrobenzoyl-L-(+)-glutamic acid	0.54	-	pale yellow
4-Aminobenzoic acid	0.73	0.77	yellow
4-Aminohippuric acid	0.34	0.45	orange
4-Aminobenzoyl-L-(+)-glutamic acid	0.33	-	yellow
4-Acetylaminobenzoic acid	0.67	0.72	none
4-Acetylaminohippuric acid	0.28	0.42	orange

A = Benzene-acetone-glacial acetic acid (2:2:1 v/v)

G = Benzene-acetone-pentan-1-ol-glacial acetic acid (1:1:1:1 v/v)

(vi) Indol-3-ylacetic acid.

Indol-3-yl[*carboxy*-^{14}C]acetic acid (specific activity 47.2 mCi/mmol) was purchased from the Radiochemical Centre, Amersham, U.K. Indol-3-ylacetic acid (m.p. 165-169^{0}C) was purchased from the Aldrich Chemical Company Inc., U.K.

Indol-3-ylacetylglycine (m.p. 78°C) was available in the department. The synthesis of indol-3-ylacetyltaurine has been described. R_F values of indol-3-ylacetic acid and some possible metabolites are given in Table 2.9.

Table 2.9 Thin-Layer Chromatography of Indol-3-ylacetic Acid and some Possible Metabolites.

Method as described in Table 2.3.
Chromatograms were sprayed with Ehrlich's reagent.

Compound	R_F values in system			Colour
	D	E	F	reaction
Indol-3-ylacetic acid	0.54	0.97	0.74	blue
Indol-3-ylacetylglycine	0.00	0.86	0.58	blue
Indol-3-ylacetyltaurine	0.00	0.67	0.40	blue

D = Chloroform-methanol (4:1 v/v)
E = Butan-2-one-1% aqueous sodium acetate-glacial acetic acid (10:5:2 v/v)
F = Butan-1-ol-glacial acetic acid-water (4:1:2 v/v)

(vii) 1-Naphthylacetic acid.

1-Naphthyl[*carboxy*-^{14}C]acetic acid (specific activity 54.5 mCi/mmol) was obtained from the Radiochemical Centre, Amersham, U.K. 1-Naphthylacetic acid (m.p. 129-131°C) was purchased from the Aldrich Chemical Company, Inc., U.K. 1-Naphthylacetylglycine (m.p. 149-151°C), 1-naphthylacetyl-L-(+)-glutamine (m.p. 172-175°C) and 1-naphthylacetyl-β-D

glucuronide (m.p. 170-172°C (d)) were kindly supplied by my colleague Frank Dixon. The synthesis of 1-naphthylacetyltaurine has been described. The R_F values of 1-naphthylacetic acid and some possible metabolites are given in Table 2.10.

(viii) 2-Naphthylacetic acid.

2-Naphthyl[*carboxy*-^{14}C]acetic acid (specific activity 0.64 mCi/mmol) was synthesized and kindly supplied by Frank Dixon together with 2-naphthylacetylglycine (m.p. 148-150°C), 2-naphthylacetyl-L-(+)-glutamine (m.p. 162°C) and 2-naphthylacetyltaurine (m.p. 280°C (d)). 2-Naphthylacetic acid (m.p. 143°C) was purchased from the Aldrich Chemical Company, Inc., U.K. The R_F values of 2-naphthylacetic acid and some possible metabolites are given in Table 2.11.

(iv) Diphenylacetic acid.

Diphenyl[*carboxy*-^{14}C]acetic acid (specific activity 0.19 mCi/mmol) was synthesized and supplied by Frank Dixon together with diphenylacetylglycine (m.p. 141-142°C), diphenylacetyltaurine (m.p. 200-205°C) and diphenylacetyl-β-D-glucuronide (m.p. 175°C). Diphenylacetic acid (m.p. 148°C) was purchased from the Aldrich Chemical Company, Inc., U.K. The R_F values of diphenylacetic acid and some possible metabolites are given in Table 2.12.

(x) 2-Phenylpropionic acid (Hydratropic acid).

(±)-2-Phenyl[3-^{14}C]propionic acid (specific activity 0.032 mCi/mmol) was synthesized and supplied by Frank Dixon together with (±)-2-phenylpropionylglycine (m.p. 102-103°C), (±)-2-phenylpropionyl-L-(+)-glutamine (m.p. 139-140°C) and

Table 2.10 Thin-Layer Chromatography of 1-Naphthylacetic Acid and some Possible Metabolites.

Method as described in Table 2.3.

Compound	R_F values in system			
	A	C	H	J
1-Naphthylacetic acid	0.77	0.99	0.85	0.86
1-Naphthylacetylglycine	0.55	0.73	0.50	0.59
1-Naphthylacetyl-L-(+)-glutamine	0.36	-	-	-
1-Naphthylacetyltaurine	0.19	0.13	0.18	0.96
1-Naphthylacetyl-β-D-glucuronide	-	-	0.00	-

Table 2.11 Thin-Layer Chromatography of 2-Naphthylacetic Acid and some Possible Metabolites.

Method as described in Table 2.3.

Compound	R_F values in system	
	A	C
2-Naphthylacetic acid	0.79	0.99
2-Naphthylacetylglycine	0.53	0.71
2-Naphthylacetyl-L-(+)-glutamine	0.35	-
2-Naphthylacetyltaurine	0.14	0.11

A = Benzene-acetone-glacial acetic acid (2:2:1 v/v)

C = Acetone-benzene-pentan-1-ol-glacial acetic acid (5:4:2:1 v/v)

H = Benzene-methanol-glacial acetic acid (6:1:1 v/v)

J = Benzene-methanol (1:1 v/v)

(±)-2-phenylpropionyltaurine (m.p. 88-89°C). (±)-2-phenylpropionic acid (b.p. 269-262°C) was purchased from the Aldrich Chemical Company, Inc., U.K. The R_F values of 2-phenylpropionic acid and some possible metabolites are given in Table 2.13.

Table 2.12 Thin-Layer Chromatography of Diphenylacetic Acid and some Possible Metabolites.

Method as described in Table 2.3.

Compound	R_F values in system	
	B	K
Diphenylacetic acid	0.65	0.93
Diphenylacetylglycine	0.34	0.84
Diphenylacetyltaurine	0.03	0.65
Diphenylacetyl-β-D-glucuronide	-	0.15

Table 2.13 Thin-Layer Chromatography of 2-Phenylpropionic Acid and some Possible Metabolites.

Method as described in Table 2.3.

Compound	R_F values in system	
	A	B
(±)-2-Phenylpropionic acid	0.80	0.74
(±)-2-Phenylpropionylglycine	0.59	0.39
(±)-2-Phenylpropionyl-L-(+)-glutamine	0.39	0.13
(±)-2-Phenylpropionyltaurine	0.19	0.03

A = Benzene-acetone-glacial acetic acid (2:2:1 v/v)

B = Benzene-acetone-glacial acetic acid (6:2:1 v/v)

K = Chloroform-methanol-glacial acetic acid (24:8:1 v/v)

Animals.

(i) ferrets

Both mongrel albino ferrets and polecat ferrets (both *Mustela furo*, see Ewer (1973)) were purchased from local dealers and a breeding colony established. Only healthy female ferrets (body weight 0.5-1.2kg) were used for experiment. Ferrets were maintained on a diet of tinned meat and allowed water *ad libitum*. Animals on such a diet were found to produce gelatinous faeces which could not be separated from the urine. To facilitate differentiation of urine during experiments, the ferrets were starved 24h prior to experiment and then fed dead mice (two daily). This had the effect of producing tightly-bound faecal pellets. For experiments, the animals were housed separately in cages mounted on stainless steel funnels for the collection of urine. For intraperitoneal administration (i.p.), compounds were given as their sodium salts in water (1-2ml) by mid-line injection into the peritoneum using a No.12 hypodermic needle (23G x 30mm). For oral administration (p.o.), compounds were injected sub-cutaneously into freshly-killed mice which were bolted by the ferrets. Urine was collected for 24h by filtration through a small glass wool plug. Cages and funnels were thoroughly washed with distilled water and the washings added to the urine. Subsequent collections were made if the 24h recovery of ^{14}C in urine was low (arbitrarily <65%). Urines were frozen and stored at $-20^{0}C$ until required.

(ii) cats

Domestic cats (*Felis catus*) were available within the department. Both female and neutered male cats (body weight

3-4kg) were used. They were maintained, caged and injected (3-5ml dose solution) similarly to ferrets, but were fed tinned meat and not mice during experiments.

(iii) dogs

4 female domestic dogs (*Canis familiaris*; body weight 13-16kg) were obtained from a local dealer. Three mongrels and one whippet were used. They were maintained on a diet of biscuits and tinned meat and were allowed water *ad libitum*. During experiments they were caged similarly to ferrets and compounds were administered i.p. as their sodium salts in water (4-10ml) by mid-line injection using a No.1 hypodermic needle (21G x 40mm). Due to its low solubility in water, 1-naphthylacetic acid was given to dogs orally as a powder in gelatine capsules concealed in pieces of meat which had been injected with the radioactive solution. Collection and storage of urine was performed as described for the ferret.

(iv) rabbits

Female Dutch rabbits (*Lepus cuniculus*; body weight 2.2-2.8kg) were obtained from a local dealer and maintained on Labsure Diet RAF (Christopher Hill Ltd., Poole, U.K.) and allowed water *ad libitum*. Experiments were performed similarly to those on ferrets.

(v) hamsters

Female Syrian golden hamsters (*Mesocricetus auratus*; body weight 0.10kg) were obtained from a local dealer and maintained on a diet of Labsure Diet 41B Modified Nuts (Christopher Hill Ltd., Poole, U.K.). During experiments they were housed separately in all-glass metabolism cages designed

for the separate collection of urine and faeces ("Metabowls"; Jencons Scientific Ltd., Hemel Hempstead, U.K.). Compounds were administered i.p. by mid-line injection of their sodium salts in water (1.5ml) using a No.20 hypodermic needle (25G x 15mm). Urine was collected directly into small conical flasks cooled with solid CO_2 ("Drikold") in insulated polystyrene boxes. Rapid freezing of urine occurred, and it was hoped that this would prevent both chemical and bacterial hydrolysis of labile metabolites such as ester glucuronides.

(vi) rats

Female Wistar albino rats (*Rattus norvegicus*; body weight 0.20kg) were used. They were maintained, caged and injected similarly to hamsters. Urine was collected similarly to ferrets.

(vii) pigeons

Both male and female pigeons (*Columba livia*; body weight 0.38-0.45kg) were obtained from a local dealer. On arrival, they were screened for *Cryptococcus neoformans*, (Mycology Department, St. Mary's Hospital) by examination of fresh and aged excreta. Pigeons were also screened for *Psittacosis* by withdrawal of 1ml blood from the wing vein and subsequent titration of antibody (Diagnostic Virology Unit, St. Mary's Hospital). No *Cryptococcus* (a fungus pathogenic to man causing cryptococcal meningitis; carried by pigeons) was found, but a range of *Psittacosis* titres from 1/10 to 1/160 was found. Animals with a titre <1/20 (arbitrary) were destroyed by decapitation and the carcasses disposed of in an incinerator. Handling of pigeons was also restricted to three persons from whom serum samples were taken and stored. With the development

of any respiratory symptoms in these workers, future serum samples could be titrated for *Psittacosis* antibody and compared to the control levels. For the handling and homogenization of pigeon excreta, workers always wore gloves and face masks, and carried out all work in an efficient fume cupboard.

Pigeons were maintained on a diet of grain and water *ad libitum*. They were housed in cages mounted on trays with a polythene lining on which a fine layer of sand (acid-purified sand, 30-90 mesh, 80g; Fisons Scientific Apparatus, Loughborough, U.K.) had been sprinkled for the collection of excreta. It was found that excreta adsorbed onto sand in this way could be scraped quantitatively off the polythene lining, which gave a better recovery of ^{14}C in excreta than in a previous method (James, 1972). Pigeons were injected i.p. with compounds as their sodium salts in water (1-2ml). The sand-excreta mixture was suspended in water (100-150ml), homogenized to dissolve radioactive metabolites and then centrifuged. The clear supernatant was frozen and stored at $-20^{0}C$ until required.

(viii) hens

Day-old chicks (sex unknown; White Leghorn or Rhode Island Red crossed with Light Sussex) were the gift of the Department of Virology, St. Mary's Hospital. They were reared and maintained on a diet of grain and allowed water *ad libitum*. The hens (*Gallus domesticus*) were used for experiment between one month (body weight 0.25kg) and four months old (1.0-1.6kg) and were caged and injected and excreta collected similarly to pigeons.

(ix) fruit bats

Two species of fruit bat were studied:

a) Indian fruit bats (*Pteropus giganteus*) were available within the department. 3 bats were used, 2 female (body weight 0.37-0.39kg) and 1 male (0.81kg). Since these animals were caught in the wild, it was not possible to estimate their age. They were maintained as described by Bababunmi *et al.* (1973). Compounds were administered i.p. as their sodium salts in water (2-3ml) and urine collected for 24h by filtration through a small plug of glass wool. Since these animals have furry coats and hang upside-down, careful body washing was performed to obtain a good recovery of ^{14}C. Body and cage washings were combined with the urine, frozen and stored at -20°C until required.

b) Singing fruit bats(Franquet's fruit bat, *Epomops franqueti*) were administered [^{14}C]benzoic acid at the University of Ibadan, Nigeria. Urine was collected into trays containing $HgCl_2$. The volume of combined urine and washings was measured and this was then adjusted to pH6 with glacial acetic acid and transported by air to St. Mary's Hospital Medical School for analysis. On arrival, the urines were frozen and stored at -20°C until further required.

Determination of Recovery.

Recovery of ^{14}C in urine was determined by scintillation counting. Urine (0.1-1.0ml) was counted in duplicate in vials containing a dioxan-based scintillation cocktail (5-20ml; Bridges *et al.*, 1967) using a scintillation spectrophotometer (Packard models 3214 and 3320). Vials were counted after sufficient time had elapsed for cooling in the

instrument (0-5°C, model 3214; 10°C, model 3320). Quench correction was performed by the method of "channels ratio". Vials were recounted several days later to allow for chemiluminescence effects.

Radiochromatographic Procedures.

% ^{14}C in urine as various metabolites was determined by radiochromatography and reverse isotope dilution. Analysis of urine from ferrets given 4-chlorophenylacetic acid was performed by paper chromatography using Whatman 3mm paper. Urine (0.1-0.5ml) was applied as a band to a paper strip (500 x 50mm) and the chromatogram developed in a suitable solvent (see Table 2.4) which was allowed to descend approximately 400mm. The paper was dried at room temperature and scanned to locate areas of radioactivity using a radiochromatogram scanner (Packard model 7200). The chromatogram was cut laterally into small sections (10mm) which were counted in the dioxan-based scintillator cocktail as described (*vide supra*). Peaks were identified by comparison of R_F values in several systems with various standards, and by their colour reactions, and quantitated from the scintillation counting results.

Analysis of urine from all other experiments was performed by thin-layer chromatography (t.l.c.) using aluminium-backed silica gel 60 F_{254} t.l.c. plates (Merck, Darmstadt, W.Germany). Urine (0.01-0.10ml) was applied as a band to a piece of plate (200 x 10-50mm) and the chromatogram developed in a suitable solvent which was allowed to run 150mm. Plates were dried under a current of cold air and then taped onto glass plates (200 x 50mm) prior to radiochromatogram scanning. Areas of the chromatograms corresponding to radio-

active peaks were carefully cut out and the whole aluminium and silica gel transferred into scintillation vials for counting. Radioactive peaks were identified by co-chromatography with various standards in several systems. Where possible, confirmation of metabolites was performed by reverse isotope dilution techniques. When such methods failed to identify a metabolite, the chromatographic band was generally purified further, isolated and various tests performed upon it in an attempt to elucidate its nature. Glucuronides were identified by indirect methods involving hydrolysis of the ester glycosidic linkage with both β-glucuronidase and dilute alkali, and chromatographically examining the aglycone. Chromatograms were also sprayed with naphthoresorcinol (5:1 mixture of 0.2% w/v naphthoresorcinol in ethanol : 85% orthophosphoric acid; glucuronic acid derivatives gave blue spots on a pink background after heating at 100°C for 10min) to confirm the presence of a glucuronide.

In cases where the radioactivity in urine was too low to permit t.l.c and radiochromatogram scanning of neat urine ($<10^5$dpm/ml), two alternative methods of concentration were used. Firstly, urine (1 vol) was lyophilized and the dry residue extracted with MeOH (0.1-0.2 vol). This effected a 5- to 10-fold concentration of the ^{14}C in urine. Secondly, ^{14}C-metabolites were extracted from urine using a column of Amberlite XAD-2 resin (BDH Chemicals Ltd., Poole, U.K.) essentially as described by Mulé *et al.* (1971). The eluate from this method could be concentrated by evaporation of the MeOH under a stream of N_2 at room temperature. The latter method was found to give the cleanest extract. Straight solvent extraction of urine was found to be unsatisfactory since highly polar metabolites such as glucuronides and

taurine conjugates were not easily extractable, even at pH 1-2.

Reverse Isotope Dilution (r.i.d.) Procedures.

(i) Benzoyltaurine

R.i.d. for benzoyltaurine was performed on isolated t.l.c. bands and not with neat urine. Urine from ferrets given [^{14}C]benzoic acid was subjected to t.l.c. as described. The band corresponding to the peak of lowest R_F (0.15 in system A) was cut from the chromatogram and eluted with MeOH (20-30ml) and filtered to remove silica gel. Carrier was added (500mg) and dissolved by boiling the solution, which was then cooled in ice and sufficient acetone added to precipitate the benzoyltaurine. Benzoyltaurine was recrystallized from methanol-acetone mixtures to constant specific activity (m.p. 295-300°C).

(ii) Hippuric acid.

R.i.d. for hippuric acid was carried out as described by Adamson *et al.* (1970*a*).

(iii) Benzoyl-L-(+)-glutamic acid.

Urine from bats given [^{14}C]benzoic acid was analysed for benzoyl-L-(+)-glutamic acid. The urine (10ml) was saturated with NaCl and then benzoyl-L-(+)-glutamic acid (1g) added, dissolution being facilitated with a little $NaHCO_3$. The solution was brought to pH1 with 11.6M-HCl and kept for 30min. It was then extracted with ethyl acetate (3 x 50ml). The bulked extracts were dried by filtering through Whatman Phase Separating (P.S.) paper and then reduced to 1-2ml at 40°C in a rotary evaporator. Boiling ethanol (25ml) followed by benzylamine (3ml) was added. The solution was cooled to

-10°C and treated with acetone (30ml), followed by light petroleum, b.p. 40-60°C (100ml). A white precipitate separated, which was filtered off, triturated with light petroleum and dried *in vacuo*. The *benzylamine salt* of benzoyl-L-(+)-glutamic acid was recrystallized to constant specific activity from ethanol-water (20:1 v/v). It had a m.p. of 182-184°C. (Found: C, 63.65; H, 6.2; N,7.9%. $C_{19}H_{22}N_2O_5$ requires C, 63.7; H, 6.2; N, 7.8%).

(iv) Phenylacetyl-L-(+)-glutamine.

Urine from ferrets given [^{14}C]phenylacetic acid was analysed for phenylacetyl-L-(+)-glutamine by r.i.d. Carrier (1g) was added to urine (5ml), warmed to dissolve, left to equilibrate for 30min and then freeze-dried. Anhydrous sodium sulphate (5-10g) was added to the yellow sticky residue and the mixture allowed to stand in a stoppered flask for 3 days. The hard solid residue was comminuted and extracted in a soxhlet with ethyl acetate. The solution was reduced to dryness in a rotary evaporator and the white residue recrystallized to constant specific activity from ethyl acetate. It was observed that this material had a higher m.p. (105-106°C) than the synthetic carrier (101°C). The solubility of the extracted material was considerably lower (<1%) than the synthetic carrier (20-25%). This suggested that the extracted material was more polar than the synthetic carrier and was possibly some salt or complex witn a urinary component. Thierfelder & Sherwin (1914) first observed the complexing of phenylacetylglutamine with urea in human urine, and this was subsequently confirmed by James (1972) in man and other species. Several tests showed that the carrier extracted from ferret urine was a 1:1 complex between phenylacetylglutamine and urea (see

Chapter 3). The extracted complex of constant specific activity was nitrated according to James (1972) and the resulting 4-nitrophenylacetylglutamine recrystallized to constant specific activity from water (m.p. 185-186°C).

(v) 4-Hydroxyphenylacetic acid.

Urines from ferrets and hamsters given [^{14}C]phenylacetic acid were analysed for 4-hydroxyphenylacetic acid by r.i.d. Carrier (1g) was added to urine (5ml) and warmed to dissolve. White needles separated on standing, which were recrystallized from hot water to constant specific activity (m.p. 149-151°C).

Hydrolysis Techniques.

Hydrolysis of conjugates was performed in 3 ways: total acid hydrolysis, mild alkali hydrolysis and enzymic hydrolysis.

(i) Total acid hydrolysis.

Amide and ester bonds formed by the conjugation of aromatic acids with amino acids and glucuronic acid were cleaved by prolonged hydrolysis with conc. HCl. Urine or an aqueous solution of an extracted metabolite (1ml) and 11.6 M-HCl (1ml) were sealed in a thick-walled glass tube and heated in an oven at 110-120°C for 15-20h. The tube was cooled in an ice-bath, opened cautiously and the contents dried *in vacuo* over NaOH pellets. The dry residue was dissolved in distilled water (0.5ml) and this solution used for t.l.c. and/or amino acid analysis. It was found that if the hydrolysate was neutralized with $NaHCO_3$ instead of drying *in vacuo*, proper amino acid chromatography could not be performed due to the high concentration of NaCl.

(ii) Mild alkali hydrolysis.

To urine (1ml) was added 2M-NaOH (1ml) and the mixture

heated in a boiling water bath for 30min using test tubes fitted with glass marbles to prevent evaporation of the contents. The solution was cooled, neutralized to phenolphthalein with 11.6M-HCl and used for t.l.c. It was found that under these conditions only the labile ester glucuronides were hydrolysed and not the more alkali-stable amino acid conjugates.

(iii) Enzymic hydrolysis.

Urine samples (0.1ml) were adjusted to pH 5 with 0.2M acetate buffer (0.4ml) and β-glucuronidase solution (0.5ml; Ketodase, Warner and Co. Ltd., Eastleigh, U.K.) added. The solutions were incubated at 37°C for 15-20h, MeOH (1-2ml) added and the denatured enzyme removed by centrifugation. The supernatant was used for t.l.c. Control tubes were set up using phenolphthalein-β-D-glucuronide Na salt (Koch-Light, Colnbrook, U.K.) as substrate and saccharic acid-1,4-lactone (Sigma Chemical Company Ltd., Kingston-upon-Thames, U.K.) as inhibitor. β-Glucuronidase was found to have no observed effect on the various amino acid conjugates.

Amino Acid Chromatography.

3 methods were employed: t.l.c., ascending paper chromatography and ion-exchange chromatography on an automatic amino acid analyser.

(i) Paper chromatography.

The systems used were:

L = butan-1-ol-glacial acetic acid-water (4:1:1 v/v)

M = propan-1-ol-ammonia (specific gravity 0.88) (7.3 v/v)

N = butan-1-ol-glacial acetic acid-water (12:3:5 v/v)

P = phenol-water (4:1 w/v)

Q = ethanol-glacial acetic acid-water (18:1: v/v)

Systems L,M and Q were used with Whatman No.4 paper and systems N and P with Whatman No.1 paper. Amino acids were visualized as blue-purple spots after spraying with ninhydrin (0.1% in acetone). The R_F values of some amino acids are given in Table 2.14.

Table 2.14 Paper Chromatography of some Amino Acids.

	R_F in system				
Amino acid	L	M	N	P	Q
Glutamic acid	0.29	0.14	0.33	0.29	0.09
Aspartic acid	0.20	0.14	0.24	0.16	0.08
Glycine	0.25	0.38	-	-	0.26
Taurine	0.17	0.48	-	-	0.36
Alanine	0.34	0.50	-	-	-

(ii) Thin-layer chromatography.

Hydrolysates of isolated metabolites were analysed for amino acids by t.l.c. using silica gel 60 F_{254} plates. The R_F values of some amino acids are given in Table 2.15. With t.l.c., greater variation of colour was observed with ninhydrin spray than was observed on spraying paper chromatograms.

(iii) Automatic amino acid analysis.

Analysis was performed using a Locarte amino acid analyser. 4-Fluorophenylalanine (PFPA) was used as internal standard. A discontinuous buffer gradient (pH 3.25, 110min; pH 4.25, 110min; ph 6.65, 160min) was used to eluate the amino acids off the column. The instrument measured amino acids

by a quantitative ninhydrin reaction both at 435 and 570nm. The retention times of certain amino acids are given in Table 2.16.

Table 2.15 Thin-Layer Chromatography of some Amino Acids.

	R_F in system		Colour reaction †	
Amino acid	M	P	M	P
Taurine	0.36	0.18	Purple	Maroon
Glycine	0.26	0.18	Purple	Orange/red
Proline	0.37	0.51	Red	Yellow
Arginine	0.10	0.03	Purple	Purple
Glutamic acid	0.15	0.14	Purple	Purple
Aspartic acid	0.13	0.10	Blue	Pale blue

M = Propan-1-ol-ammonia (specific gravity 0.88) (7:3 v/v)

P = Phenol-water (4:1 w/v)

† Sprayed with ninhydrin (0.1% in acetone) and heated with a hot air blower for 30sec. Colours differed depending on the t.l.c. system used.

Table 2.16 Retention times of some Amino Acids on a Locarte Analyser.

	Retention	
Amino acid	measured (min)	relative†
Group 1 (pH 3.25 buffer)		
Aspartic acid	42	0.47
Threonine	50	0.55
Serine	53	0.58
Glutamic acid	68	0.75
Proline	73	0.81
Glycine	90	1.00
Alanine	100	1.10

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Table 2.16 Contd.

Group 2 (pH 4.25 buffer)		
1/2 Cystine	126	0.62
Valine	130	0.64
Methionine	141	0.69
Isoleucine	151	0.75
Leucine	160	0.79
Tyrosine	187	0.92
Phenylalanine	197	0.97
PFPA	203	1.00
Group 3 (pH 6.65 buffer)		
Histidine	233	0.94
Lysine	242	0.97
Ammonia	249	1.00

† Group 1 measured relative to glycine.
Group 2 measured relative to PFPA.
Group 3 measured relative to ammonia.

Quantitative Amino Acid Determination.

Hyrolysates from certain isolated metabolites were subjected to quantitative ninhydrin amino acid determinations. 2 methods were used, one using stannous chloride as reducing agent and the other sodium cyanide.

(i) To the amino acid solution (1ml; 0-100µg) was added a 1:1 mixture of a solution of ninhydrin in methyl-cellosolve (2% w/v) and 0.5M citrate buffer (pH 5) which contained 0.1% stannous chloride (1ml). The solution was heated in a boiling-water bath for 20min, cooled and

propan-1-ol-water mixture (1:1 v/v; 5ml) added. The solution was allowed to stand for 10min and then the optical density read at 570nm using a Unicam SP500 or SP600 spectrophotometer. This method obeyed a linear relationship for various amino acids (0-250nmol).

(ii) The second method is essentially that of Rosen (1957) which used the following solutions:

(a) 0.1M-NaCN

(b) strong pH 5 acetate buffer [sodium acetate trihydrate (270g) + glacial acetic acid (50ml) made up to 750ml]

(c) 3% ninhydrin in methylcellosolve

(d) propan-2-ol-water (1:1 v/v)

Solution (a) (0.2ml) and solution (b) (9.8ml) were mixed just prior to the experiment, and to an aliquot of this solution (0.25ml) was added the amino acid solution (0.25ml) followed by solution (c) (0.25ml). The mixture was heated at 100°C in a boiling-water bath for 15min, cooled and solution (d) (2.5ml) added. The blue colour was read at 570nm. This method was found to give a linear calibration for glutamic acid (0-100nmol).

Detection of Phenylacetyl-L-(+)-glutamine in Ferret Urine.

Ferret urine (20ml) was saturated with NaCl, acidified to pH 1 with 11.6M-HCl and extracted with dichloromethane (3x50ml). The bulked extracts were dried by filtration through Whatman PS paper and reduced to a small volume (<1ml) in a rotary evaporator. The residue was chromatographed on t.l.c. (plates 200 x 200mm) in system A. A small section of each plate was scanned to locate the radioactive bands and

the area corresponding to phenylacetylglutamine (R_F 0.39) was cut out and eluted with MeOH (20ml). The MeOH eluate was taken to dryness under a stream of N_2 and the residue dissolved in distilled water (0.5ml). A small aliquot was chromatographed and ran as a single spot (R_F 0.39). Total acid hydrolysis was performed and the hydrolysate subjected to automatic amino acid analysis. Five peaks were found, which were identified as aspartic acid (t = 0.49 relative to glycine; 15.5% total amino acids), serine (t = 0.60; 0.4%), glutamic acid (t = 0.76; 69.8%), glycine (t = 1.00; 1.6%) and ornithine or histidine (t = 0.95 relative to ammonia; 12.8%). When the band from the chromatogram corresponding to phenylacetylglycine was put through this procedure a single amino acid peak corresponding to glycine (100.0%) was found. Control ferret urine subjected in a similar fashion gave no detectable amino acids on the analyser. Synthetic phenylacetylglutamine was found to quantitatively yield glutamic acid and ammonia on total acid hydrolysis by measurement against a calibration curve constructed for glutamic acid-ammonia (0.5 mole fraction).

Reverse isotope dilution on a single ferret urine showed the presence of phenylacetyl-L-(+)-glutamine (5.1% of 24h urinary ^{14}C).

Detection of Benzoyl-L-(+)-glutamic Acid in Fruit Bat Urine.

Urine from Indian fruit bats given [^{14}C]benzoic acid was chromatographed in system A. The band at R_F 0.51, which corresponded to 5-16% (in 4 urines) of the ^{14}C excreted, was cut from the chromatograms and eluted with MeOH (50ml). The MeOH was removed at 40^{0}C and the white residue dissolved in distilled water (0.5ml). T.l.c. of a sample (10µl) of the aqueous solution showed that the material ran as a single substance with R_F 0.51 in solvent A, 0.18 in B and 0.60 in C,

which corresponded to benzoylglutamic acid (see Table 2.7). The aqueous solution was subjected to total acid hydrolysis and the hydrolysate analysed for amino acids by paper chromatography and colorimetry as described. The hydrolysate ran as a single spot with R_F 0.27 in system L, 0.13 in system M, o.32 in system N and 0.27 in system P, showing that it contained glutamic acid. The amount of glutamic acid in the hydrolysate was determined by a quantitative ninhydrin reaction (Rosen, 1957) against a standard curve prepared with L-(+)-glutamic acid, and was found to be 1.86µmol/ml. On t.l.c. of the hydrolysate, a single ^{14}C peak was found, with R_F 0.79 in solvent A corresponding to benzoic acid. On scintillation counting, the amount of benzoic acid present was found to be 2.20µmol/ml. This demonstrated that the metabolite with R_F 0.51 in system A contained benzoic acid and glutamic acid in a molecular ratio 1:1.

Reverse isotope dilution for benzoyl-L-(+)-glutamic acid as its benzylamine salt confirmed the presence of benzoyl-L-(+)-glutamic acid in these urines (5-15% of 24h urinary ^{14}C).

Detection of 2-Phenylpropionylglycine and 2-Phenylpropionyltaurine in Ferret Urine.

Urine from ferrets given [^{14}C]2-phenylpropionic acid (hydratropic acid) was chromatographed on thin-layer plates in system A and the bands corresponding to 2-phenylpropionylglycine (R_F 0.59) and 2-phenylpropionyltaurine (R_F 0.19) were cut out and eluted separately with MeOH (50ml). The eluates were dried under a stream of N_2 and the white residues dissolved in aliquots of distilled water (0.5ml). A portion of each (10µl) was chromatographed on t.l.c. and found to run as single ^{14}C peaks (R_F 0.59 and 0.19). Both isolated

metabolites were subjected to total acid hydrolysis and radioactivity in the hydrolysates found to be [^{14}C]2-phenylpropionic acid by t.l.c. and radiochromatogram scanning. Amino acid analysis was carried out on each hydrolysate using t.l.c. and paper chromatography as described. The hydrolysate from the proposed glycine conjugate showed a single amino acid on paper chromatography (R_F 0.37 in system M; see Table 2.14) corresponding to glycine. The hydrolysate from the proposed taurine conjugate showed a single amino acid on t.l.c. (purple spot, R_F 0.35 in system M; red spot, R_F 0.16 in system P; see Table 2.15) and paper chromatography (R_F 0.38 in system Q, R_F 0.49 in system M) corresponding to taurine.

Reverse isotope dilution was not performed for these two compounds.

Determination of Aromatic Amino Groups by Bratton and Marshall Colorimetric Assay.

4-Aminobenzoic and 4-aminophenylacetic acids in urine, after dosing the animal with the corresponding nitro acid, were determined by a modification of the method of Bratton & Marshall (1939), first developed for the estimation of sulphonamides. Urines were measured against standard curves constructed for 4-aminophenylacetic acid (1-10µg assay range; λ = 560nm) and 4-aminobenzoic acid (1-10µg; λ = 547nm) before and after mild acid hydrolysis with 2M-HCl at 100^{0}C for 1h. This gave estimates of free and total (free + acetylated) aromatic amine, and thus it was possible to calculate % dose of the acid which had been reduced and acetylated in the animal body.

Validity of the Methods.

The major technique used in this work for the separation, identification and quantitation of urinary ^{14}C metabolites was t.l.c. Good chromatographic reproducibility was found, especially with systems A and B (mixtures of acetone, benzene and acetic acid) which were designed not to age. This is in contrast to such systems as those containing butanol and acetic acid, which have a very short tank life due to the reaction to the less polar butyl acetate. The purchased aluminium-backed thin-layer plates also gave good reproducibility of R_F due to their homogeneity compared with thin-layers on glass made in this laboratory. Whenever possible, chromatography tanks with greased and flanged lids were used to prevent evaporation of the solvent, and thus changes in composition. T.l.c. was found to be a much more rapid technique (development time, 45-90min per 150mm; scanning time, 30min) than paper chromatography (development time, 7-15h per 400mm; scanning time, 40-60min). Other observed advantages were:

(a) smaller and more discrete spots were obtained, *i.e.* better resolution.

(b) acidic metabolites in acidic systems chromatographed in order of their acidity, unlike the partitioning process of paper chromatography. The position of an unidentified metabolite relative to known metabolites on t.l.c. could give initial structural information.

(c) t.l.c. is a lower cost method. Counting 40 x 10mm portions of paper chromatograms, necessary to locate large diffuse spots, is more expensive than counting 4 discrete bands (say) from a thin-layer chromatogram.

Primary identification of metabolites evolved from the co-chromatography of a ^{14}C band on t.l.c. with a known standard in several systems. When the identity of a new or unusual conjugate was inferred by its position on thin-layer, subsequent analysis by reverse isotope dilution or identification of the liberated amino acid on hydrolysis confirmed the initial inference. Since t.l.c. systems have been developed which will separate many of the possible metabolites of a compound such as phenylacetic acid or benzoic acid (see Tables 2.3 and 2.7), then this would seem to validate the methods used here for the identification of such metabolites. As a quantitative technique, radiochromatography usually gave a good correspondence with reverse isotope dilution data.

The main limitations of such methods however, are wrong assignments of ^{14}C composite peaks which remain unresolved in several systems. Only much more protracted methods capable of greater resolution and specificity, such as g.c.-m.s., would go any way to solving the problem of "hidden" metabolites.

CHAPTER THREE

Results and Preliminary Discussion

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Metabolism of Phenylacetic Acid in the Ferret, Dog, Cat, Rabbit and Hamster.

The metabolism of [^{14}C]phenylacetic acid in the ferret is shown in Table 3.1. The data of James *et al.* (1972*a*) has been included for comparison. At a dose level of 100mg/kg, 3 peaks were found on radiochromatography of the 24h urine, corresponding to unchanged acid (2% of the dose), phenylacetylglycine (43%) and phenylacetyltaurine (14%). A fourth peak (9%) which corresponded to phenylacetylglutamine in all solvent systems used was also seen (see Fig. 3.1). This peak has partially been accounted for as phenylacetyl-L-(+)-glutamine (4% of the dose in 1 animal) by reverse isotope dilution (r.i.d.) as previously described (pp. 112-113). The result of an elemental analysis on the phenylacetylglutamine extracted from ferret urine during the r.i.d. procedure is given in Table 3.2 and clearly demonstrates that this material gives a better fit for a 1:1 complex between phenylacetylglutamine and urea than for phenylacetylglutamine itself. The phenylacetylglutamine-urea complex ran as a single spot (R_F 0.39 in system A) on t.l.c. and gave a positive colour reaction (yellow) for urea on spraying with Ehrlich's reagent. Urea had an R_F 0.02 in system A. Methodological problems prevented the determination of [^{14}C]phenylacetylglutamine in other urine samples by this method. When the experiment was repeated at 100mg/kg using 30μCi of ^{14}C instead of 10.8μCi per animal, a fifth minor peak corresponding to 4-hydroxyphenylacetic acid (2.2% of the dose) was found on radiochromatogram scanning of the 24h urine. This was confirmed by r.i.d. which gave a marginally lower, but significant, result of 0.7%. It is of no great surprise that such minor metabolites can be detected

Table 3.1 24h Urinary Metabolites of [^{14}C]Phenylacetic Acid in the Ferret at Different Dose Levels.

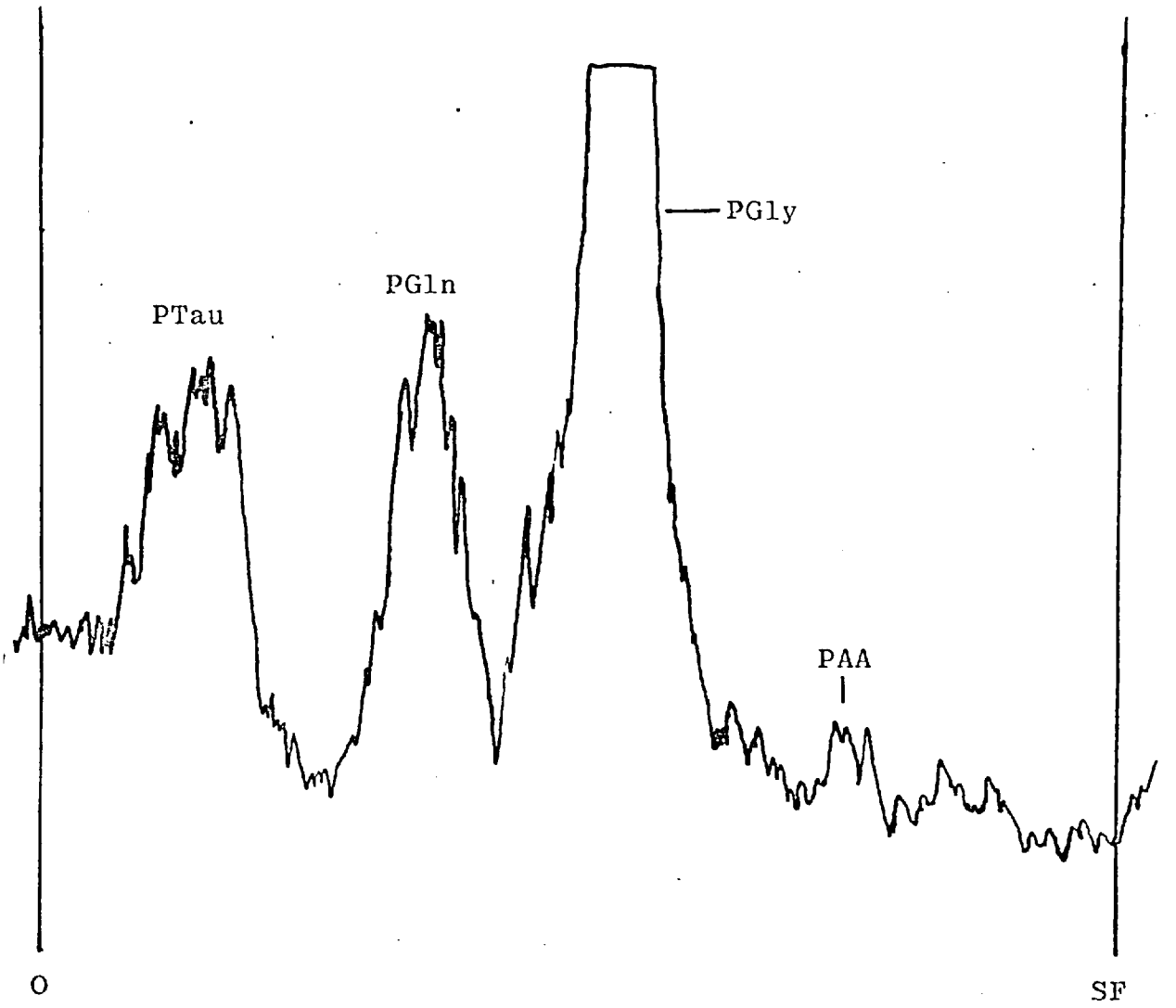
3 female ferrets were used in each experiment. The compound was administered i.p. except where stated.

Dose				% Dose Conjugated With			
mg/kg	^{14}C(μCi)	Recovery (%)	% dose unchanged	Glucuronic acid	Taurine	Glycine	Unknown
0.01	10.7	75 (55-89)	2 (1.3)	n.d.	6 (3-9)	52 (44-61)	14 (6-18)
100	10.8	68 (57-75)	2 (2-3)	n.d.	14 (14-15)	43 (34-49)	9**(7-11)
*100	30	67 (59-73)	1 (1-2)	n.d.	14 (9-17)	42 (27-46)	7 (5-7)
§100	10.1	89 (86-92)	4 (2-8)	5 (4-6)	10 (7-12)	57 (55-60)	12 (12-13)
200	10.8	69 (61-74)	3 (1-4)	3 (2-4)	26 (19-31)	28 (19-36)	8 (7-12)
400	10.8	55 (48-59)	4 (2-5)	3 (3-4)	21 (19-23)	20 (18-24)	6 (6-7)
† 80	5	95	3	21	30	41	n.d.

* 2.2 (0-2)% dose found as 4-hydroxyphenylacetic acid by radiochromatogram scanning; 0.7 (0.6-0.7)% by reverse isotope dilution.

** 4% dose found as phenylacetyl-L-(+)-glutamine in 1 urine by reverse isotope dilution; 10% by radiochromatogram scanning.

n.d. means not detected. § dose given p.o. † From James *et al.* (1972*a*).



Key to Figs.3.1 - 3.4.

PTau	phenylacetyltaurine
PGly	phenylacetylglycine
PGln	phenylacetylglutamine
PAA	phenylacetic acid
U	unidentified metabolite

Fig.3.1 Radiochromatogram Scan of Urine from a Ferret given $|^{14}C|$Phenylacetic Acid.

Table 3.2 Confirmation of 1:1 Complex Formation between Phenylacetyl-L-(+)-glutamine and Urea in Ferret Urine by Elemental Analysis.

PAgln = phenylacetylglutamine

PAgln requires	:	C, 59.1; H, 6.1; N, 10.2%
PAgln-urea (1:1) requires	:	C, 51.9; H, 6.2; N, 17.3%
PAgln-urea (2:1) requires	:	C, 46.9; H, 6.3; N, 21.9%
Found	:	C, 52.2; H, 6.2; N, 16.7%

by using larger doses of the radioisotope because a smaller amount of urine or a more dilute urine may be applied to the chromatogram, evoking a better resolution of the ^{14}C bands. Acheson & Gibbard (1962), studying the metabolism of [^{14}C]-benzoic acid by rats and guinea-pigs, gave 100-200μCi of the label to each animal and were able to show that the rat hydroxylated 0.25% of the dose (50mg/kg) and the guinea-pig 0.038% of the dose. Hydroxylation occurred to a roughly equal degree in both the *meta* and *para* positions in both species.

When ferrets were given [^{14}C]phenylacetic acid at doses of 0.01, 200 and 400mg/kg, the same qualitative pattern of metabolites was seen. However, the proportion of the dose excreted in the 24h urine which was conjugated with glycine, taurine, glucuronic acid and the unknown metabolite were found to change with dose (see Table 3.3).

While glycine conjugation diminishes with increasing dose, taurine conjugation rises markedly from 8% of the 24h urinary ^{14}C at a dose of 0.01mg/kg to 38% at 200mg/kg. Phenylacetylglucuronide does not appear until a dose level of 200mg/kg is reached. The proportion of the unidentified

metabolite remained fairly constant over the whole range (11-19%)..

Table 3.3 Quantitative Differences in the Conjugation of [^{14}C]Phenylacetic Acid at Different Dose Levels in the Ferret.

Dose (mg/kg)	Recovery of ^{14}C (%)	% ^{14}C in 24h urine conjugated with			
		glycine	taurine	glucuronic acid	unknown
0.01	75	69	8	n.d.	19
100	68	63	21	n.d.	13
200	69	41	38	4	12
400	55	36	38	5	11

n.d. means not detected.

When the compound was given orally to this species at 100mg/kg, no significant differences to the pattern of metabolism after i.p. administration were found. The only effect of route of administration appears to be the formation of the glucuronide (5% of the dose) which was not seen when the compound was given i.p. at this dose.

There are two major differences between the results of James *et al.* (1972*a*) and the results given here. The large amount of glucuronide (21% of the dose) which these workers found at 80mg/kg was not observed here, nor did they detect the peak which has been designated as phenylacetylglutamine in this study. These major differences may be methodological ones, since James *et al.* used paper chromatography, and not t.l.c., which it was found does not give as good a separation of the metabolites of aromatic acids across the full range of polarities from unchanged acid to its taurine conjugate.

The metabolism of [^{14}C]phenylacetic acid in the dog and cat is shown in Table 3.4. Both species gave a quantitative recovery of ^{14}C in the 24h urine. The major metabolite was the glycine conjugate in both species, the cat forming a small amount (1.2% of the dose) of the taurine conjugate also. This metabolite could not be detected in the dog. These findings are in keeping with the work of James *et al.* (1972*a*) who showed the glycine conjugate to be the major conjugate in both these species. Like the ferret, neither cat nor dog made the glucuronide which James *et al.* (1972*a*) reported in the dog (2% of the dose).

The metabolism of [^{14}C]phenylacetic acid in the rabbit is shown in Table 3.5. The data of James *et al.* (1972*a*) has been included for comparison. At doses of 200 and 400mg/kg, rabbits made mainly the glycine conjugate and a small amount of the glucuronide. Taurine conjugation was not observed in this species. The rabbit is not a species that might be expected to make a taurine conjugate since its hepatic and renal tissue taurine levels are very low (see Table 1.17) and it also conjugates its bile acids exclusively with glycine (Haslewood, 1967).

Table 3.4 24h Urinary Metabolites of [^{14}C]Phenylacetic Acid in the Dog and Cat.

2 female dogs and 3 female cats were dosed 100mg/kg i.p. Dogs were given 11μCi and cats 20μCi each.

Species	Recovery of ^{14}C (%)	% dose unchanged	% dose conjugated with	
			taurine	glycine
Dog	100	n.d.	n.d.	100
Cat	100	9 (8-10)	1.2(0.7-1.5)	90(89-91)

Phenylacetylglutamine and phenylacetylglucuronide were not detected in either species.

The metabolism of [14C]phenylacetic acid in the Syrian golden hamster is shown in Table 3.6. In this species, 43% of the dose was excreted unchanged in urine and 6% as a metabolite which corresponded to 3- or 4-hydroxyphenylacetic acid on t.l.c. (R_F 0.5 in system B) and was quantitatively unaffected by total acid hydrolysis, indicating that it was not a conjugate. Radiochromatogram scans of hamster urine before and after hydrolysis are given in Figs.3.2 and 3.3. The exact nature of the unidentified peak has not been elucidated, but chromatographic evidence suggests that it is a ring hydroxylated metabolite or a mixture of such metabolites. It is not labile to hydrolysis with 11.6M-HCl and thus not a conjugate bonded through an ester or amide linkage, nor is it 3,4-dihydroxyphenylacetic acid or mandelic acid (α-hydroxyphenylacetic acid) by its position on t.l.c. (see Table 2.3). The major conjugate found in the hamster was phenylacetylglycine (32% of the dose). A small amount of the taurine conjugate (1.5%) and a conjugate which co-chromatographed with phenylacetylglutamine (0.6%) were also found. T.l.c. system A was able to resolve the various conjugates, whilst system B was used to separate unchanged acid from the proposed hydroxylated metabolite. A radiochromatogram scan of hamster urine in system A is shown in Fig.3.4. This demonstrates the separation of the glycine, taurine and "glutamine" conjugates. James *et al.* (1972*a*) gave figures of 44, 40 and 0.9% of the dose for unchanged acid, glycine and taurine conjugates respectively, and in general their data are in excellent agreement with those given in Table 3.6. It is felt that their failure to detect the extra peak is again due to methodological differences.

Table 3.5 24h Urinary Metabolites of [^{14}C]Phenylacetic Acid in the Rabbit.

3 female rabbits were used in both experiments. The compound was administered i.p. (11μCi).

Dose (mg/kg)	Recovery of ^{14}C (%)	% dose unchanged	% dose conjugated with		
			glucuronic acid	taurine	glycine
200	86 (75-95)	8 (3-13)	6 (4-7)	n.d.	72 (69-76)
400	46 (41-52)	8 (6-10)	6 (3-9)	n.d.	32 (29-35)
80†	85	2	n.d.	1	83

† From James *et al.* (1972*a*).

Table 3.6 24h Urinary Metabolites of [^{14}C]Phenylacetic Acid in the Syrian Golden Hamster.

Recovery of ^{14}C (%)	% dose unchanged	% dose hydroxylated†	Total unconjugated (%)	% dose conjugated with		
				glycine	taurine	"glutamine"§
83	43	6	44	32	1.5	0.6
(75-94)	(29-52)	(3-14)	(37-57)	(25-40)	(0-4)	(0-1.8)

6 female hamsters were dosed 100mg/kg i.p. + 22μCi.

† peak which corresponded to 3- or 4-hydroxyphenylacetic acid on t.l.c.

§ peak which could not be differentiated from phenylacetylglutamine on t.l.c.

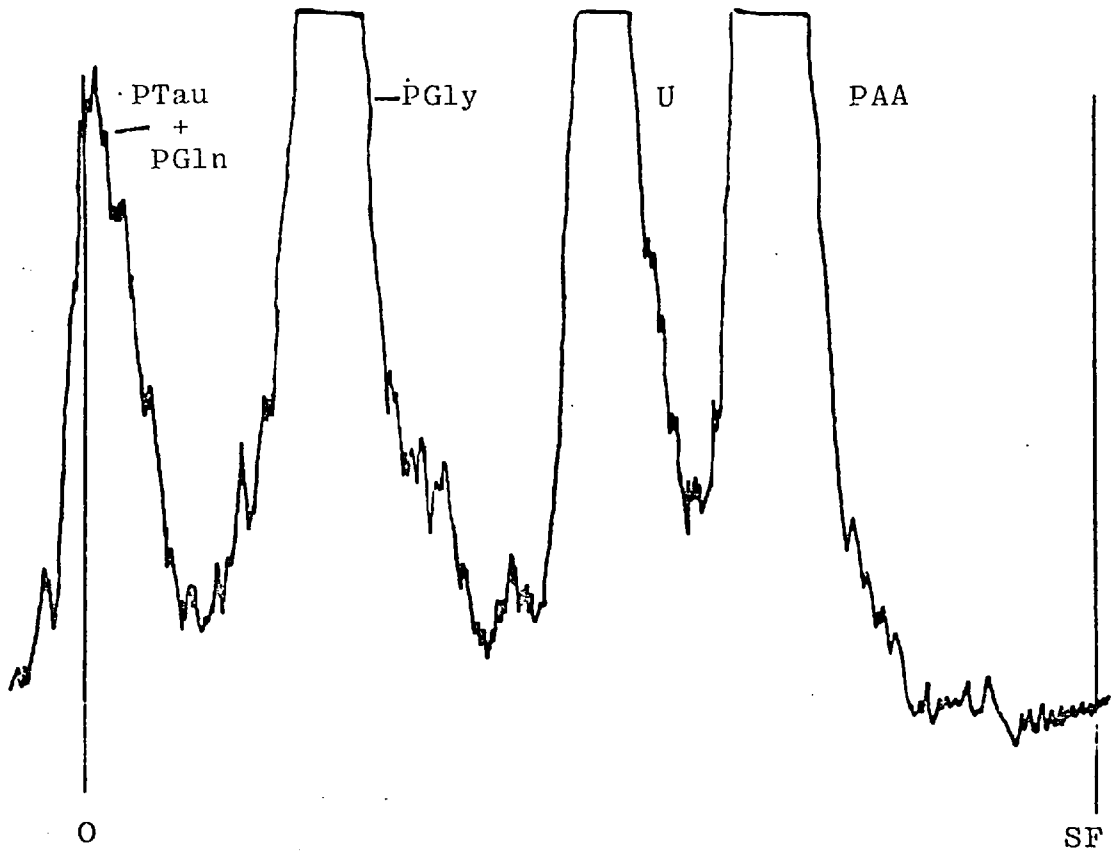
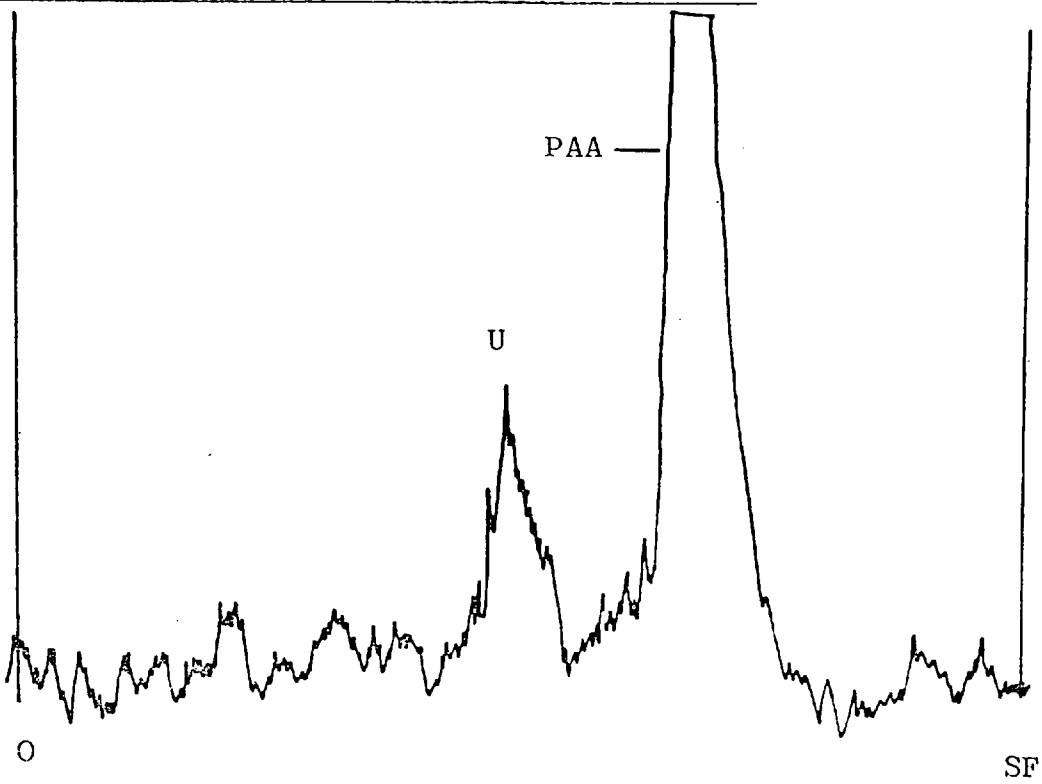
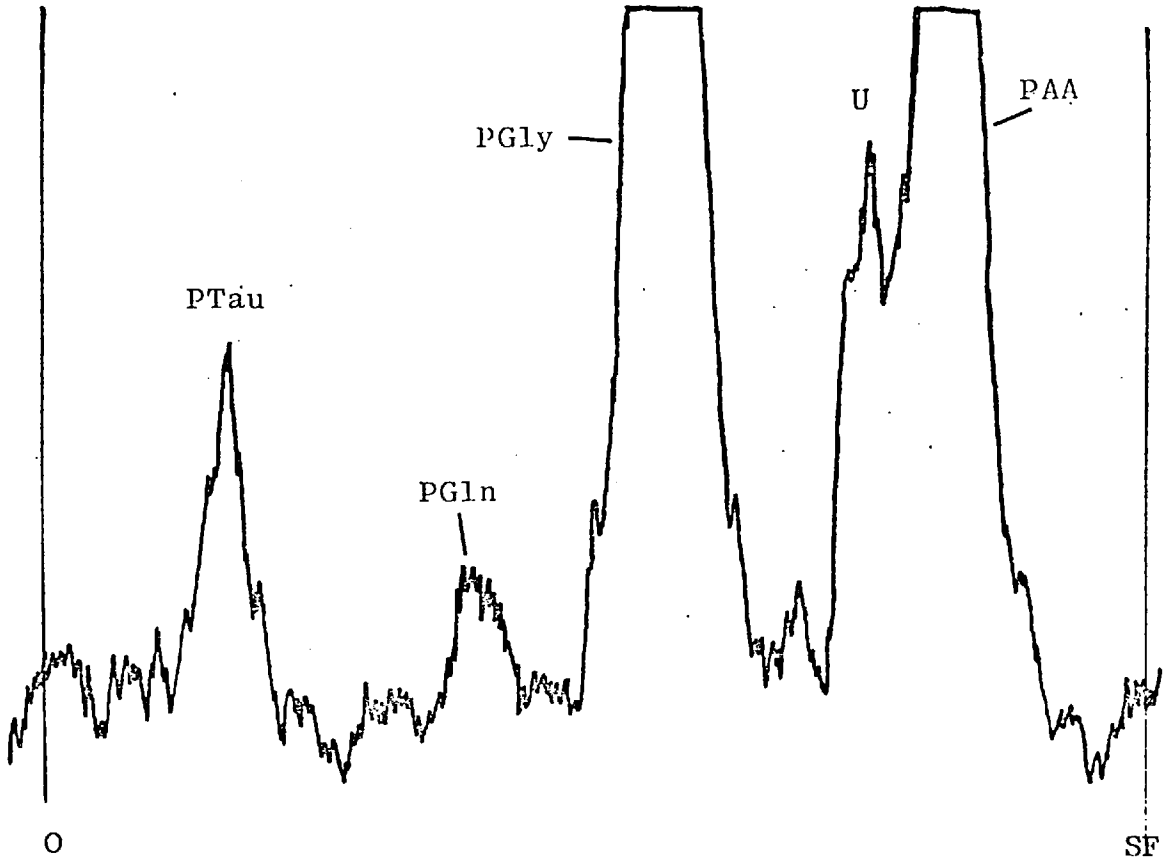


Fig.3.2 Radiochromatogram Scan (System B) of Urine from a Hamster given [^{14}C]Phenylacetic Acid.

(see page 130 for Key).

Fig.3.3 Radiochromatogram Scan of Acid-hydrolysed Urine from a Hamster given [^{14}C]Phenylacetic Acid.





(see page 130 for Key)

Fig.3.4 Radiochromatogram Scan (System A) of Urine from a Hamster given [^{14}C]Phenylacetic Acid.

Metabolism of 4-Chlorophenylacetic Acid in the Ferret, Dog and Cat.

The metabolism of [^{14}C]4-chlorophenylacetic acid in the ferret is shown in Table 3.7 and a typical radiochromatogram scan of the urine is shown in Fig.3.5. 0-3, 3-24 and 24-48h urines were collected and this was the only experiment where paper chromatography was used for analysis of metabolites. The dominant metabolite of 4-chlorophenylacetic acid was the glycine conjugate (26% of the dose in 24h), 4-chlorophenylacetyltaurine occurring as a minor metabolite (8%). It is interesting that like phenylacetic acid, 4-chlorophenylacetic acid forms a metabolite (5-8% of dose in 3 urines) in the ferret which cannot be differentiated from the glutamine conjugate by chromatography.

The metabolism of [^{14}C]4-chlorophenylacetic acid in the cat and dog are shown in Table 3.8. The compound was quantitatively recovered in the urine of both species. Urine was collected for 48h in the cats since they had not urinated on the first day. The major metabolite in both species was the glycine conjugate (dog, 76% of the dose; cat, 92%) whilst the taurine conjugate was also found, but in smaller amounts (dog, 19% of the dose; cat, 2%). 4-Chlorophenylacetylglucuronide was not found in either species, but a peak corresponding to the glutamine conjugate on t.l.c. appeared in both urines (dog, 2% of the dose; cat, 4%). Thus, the species of carnivores studied here metabolised 4-chlorophenylacetic acid in a similar fashion both qualitatively and quantitatively.

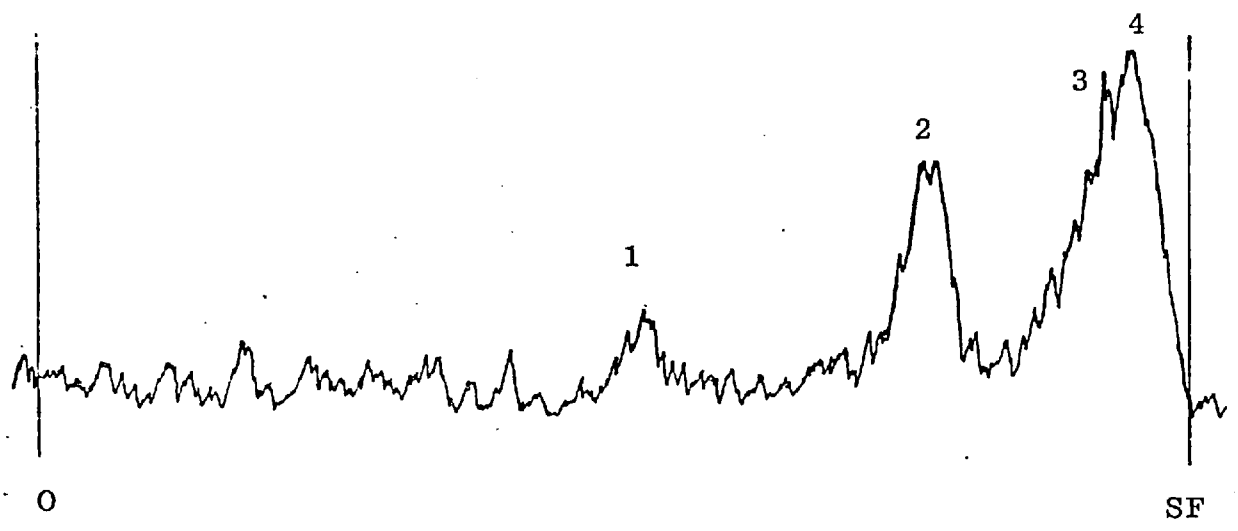
Table 3.7 Urinary Metabolites of [^{14}C]4-Chlorophenylacetic Acid in the Ferret.

3 female ferrets were dosed 100mg/kg i.p. + 5μCi.

Time (h)	Recovery (%)	% dose unchanged	% dose conjugated with glycine	% dose conjugated with taurine
0-3	31 (27-38)	18 (12-23)	7 (6-9)	6 (5-8)
0-24	87 (80-91)	47 (36-56)	26 (18-31)	8 (8-9)
0-48	90 (83-94)	47 (36-56)	28 (19-33)	8 (8-9)

4-Chlorophenylacetylglucuronide was not detected. All urines contained an unidentified metabolite which co-chromatographed with 4-chlorophenylacetylglutamine in all systems used and amounted to 5-8% of the dose.

Fig.3.5 Radiochromatogram Scan of Urine from a Ferret given [^{14}C]4-Chlorophenylacetic Acid.



1 4-chlorophenylacetylglutamine 2 4-chlorophenylacetyltaurine

3 4-chlorophenylacetylglycine 4 4-chlorophenylacetic acid

Table 3.8 Urinary Metabolites of [^{14}C]4-Chlorophenylacetic Acid in the Cat and Dog.

1 female cat, 2 neutered male cats and 2 female dogs were dosed 100mg/kg i.p.

Cats were given 11.9μCi and dogs given 9.4μCi.

Species	Duration of Experiment (days)	Recovery (%)	% dose unchanged	% dose conjugated with	
				glycine	taurine
Cat †	2	100	2 (1-4)	92 (86-98)	2 (2-3)
Dog §	1	98 (98,99)	2 (0,4)	76 (76,76)	19 (16,22)

† A peak corresponding to 4-chlorophenylacetylglutamine was found which amounted to 4% (0-7) of the dose.

§ A similar peak was found in dog urine corresponding to 2% (0,4) of the dose.

4-Chlorophenylacetylglucuronide was not detected in either species.

Metabolism of 4-Nitrophenylacetic Acid in the Ferret, Dog and Cat.

The metabolism of [^{14}C]4-nitrophenylacetic acid in the ferret is shown in Table 3.9. This compound was excreted very slowly by ferrets and only 56% of the dose was recovered in the urine in 4 days. In this case, the major metabolite was the taurine conjugate, accounting for 35% of the dose in 106h.

The metabolism of [^{14}C]4-nitrophenylacetic acid in the cat and dog is shown in Table 3.10. These two species differed in several ways. Firstly, a peak corresponding to 4-acetylaminophenylacetic acid (11% of the dose) was found on radiochromatogram scanning of cat urine. This reduced and acetylated metabolite was not found in dog urine. Bratton & Marshall colorimetric assay of cat urine (see later) confirmed this finding. Secondly, an unidentified metabolite was found in cat urine (11% of the dose) which was not found in dog urine. Furthermore, the dog excreted a large proportion (61%) of the dose unchanged. 4-Nitrophenylacetyltaurine was found in both cat (9% of the dose) and dog (15%) urine, and in the latter species it was the only conjugate. James *et al.* (1972*b*), who studied the metabolism of this compound in man, monkey and rat, did not detect any reduced metabolites although they did find an unidentified metabolite in man and rhesus monkey, which was possibly 4-nitrophenylacetyltaurine.

Metabolism of Benzoic Acid in the Ferret, Cat and Fruit Bat.

The metabolism of [^{14}C]benzoic acid in the ferret is shown in Table 3.11. This compound was well excreted by the ferret (80% of the dose in urine in 24h) and was conjugated with both glycine (34% of the dose) and glucuronic acid (51%).

Table 3.9 Urinary Metabolites of [^{14}C]4-Nitrophenylacetic Acid in the Ferret.

3 female ferrets were dosed 100mg/kg i.p. + 8.7μCi.

Time (h)	Recovery (%)	% dose unchanged	% dose conjugated with	
			glycine	taurine
0-24	22 (21-24)	6 (4-8)	2 (1-4)	14 (12-16)
0-48	41 (32-49)	9 (9-11)	4 (3-5)	28 (21-35)
0-106	56 (50-61)	14 (13-15)	8 (6-10)	35 (30-41)

4-Nitrophenylacetylglucuronide was not detected.

Table 3.10 24h Urinary Metabolites of [^{14}C] 4-Nitrophenylacetic Acid in the Cat and Dog.

3 female cats and 3 female dogs were dosed 100mg/kg i.p.

Cats were given 9.9μCi and dogs given 9.3μCi.

Species	Recovery %	% dose unchanged	% dose as		
			4-acetylamino-phenylacetic acid	4-nitrophenyl-acetyltaurine	4-nitrophenyl-acetylglycine
Cat †	56 (48-61)	22 (17-27)	11 (8-13) §	9 (8-10)	2 (1-4)
Dog	76 (71-82)	61 (60-61)	n.d.	15	n.d.

† A peak at R_F 0.04 in system G which did not correspond to any of the available standards (see Table 2.6) was found in all 3 cat urines. This metabolite amounted to 11% (9-13) of the dose.

§ Bratton and Marshall colorimetric assay (see later) gave figures of 13% (12-14) and 2% (0-5) of the dose for acetylamino and amino groups respectively.

4-Nitrophenylacetylglucuronide was not detected in either species.

Benzoyltaurine could not be detected in any urine by radiochromatographic means and this was confirmed by reverse isotope dilution. The results accord with those of Bridges *et al.* (1970) who found only the glycine and glucuronic acid conjugates of benzoic acid in the ferret. They also established that increased doses of benzoic acid in the ferret gave rise to increased conjugation with glucuronic acid.

The metabolism of [^{14}C]benzoic acid in the cat is shown in Table 3.12. In this species only the glycine conjugate was found. There was no evidence for taurine or glucuronic acid conjugation with this acid in the cat, which is known to be poor at forming glucuronides with many types of substrate (Robinson & Williams, 1958). Bridges *et al.* (1970) also showed that benzoic acid was metabolised only to hippuric acid at a dose of 51mg/kg p.o.

The metabolism of [^{14}C]benzoic acid in two species of fruit bat, *Pteropus giganteus*, the Indian fruit bat and *Epomops franqueti*, Franquet's fruit bat, has been studied and the results are shown in Tables 3.13 and 3.14. The Indian fruit bat was investigated because several workers had shown that this species of bat did not make hippuric acid from administered benzoic acid (Bridges *et al.*, 1970; Bababunmi *et al.*, 1973; Ette *et al.*, 1974). It was thought that in the absence of glycine conjugation, the Indian fruit bat might synthesize benzoyltaurine or another amino acid conjugate, and that this would shed some light upon the reported "defect" of hippuric acid synthesis in this bat. In all urines, a peak was found which corresponded to hippuric acid by t.l.c. (see Fig.3.6). However, reverse isotope dilution showed that this metabolite was not hippuric acid. Benzoyl-L-(+)-glutamic acid was found to be chromatographically similar to hippuric acid

(see Table 2.7) and so the urines were analysed for the glutamic acid conjugate by reverse isotope dilution. This technique accounted for all the ^{14}C under the "hippuric acid" peak as benzoyl-L-(+)-glutamic acid (see Table 3.13). Glutamic acid conjugation has not previously been described for vertebrates and is only known in arachnids and millipedes (Smith, 1968).

Franquet's fruit bat (or the Singing fruit bat) is a species of African fruit bat which is found, amongst other places, on or around the campus of the University of Ibadan, Nigeria. This bat has also been examined for hippuric acid synthesis. The results are given in Table 3.14 and a radiochromatogram scan is shown in Fig.3.7. 19-100% of the ^{14}C was recovered in the urine of these bats in 24h. On most chromatograms, three peaks were seen, corresponding to benzoic acid, hippuric acid (+ benzoylglutamic acid) and benzoylglucuronide. In one urine, a fourth peak (14% of urinary ^{14}C) was found whose nature has not been further investigated. Urines were analysed for both hippuric acid and benzoylglutamic acid by r.i.d. and the results of these analyses accounted for all the ^{14}C under the "hippuric acid" peak. Table 3.14 shows that this species of bat conjugated benzoic acid with glucuronic acid (32% of urinary ^{14}C) and with both glycine (31%) and glutamic acid (11%), two conjugates which could not be resolved by t.l.c., appearing as a single radioactive peak (41% of urinary ^{14}C). The further implications of these results will be discussed in Chapter Four.

Table 3.11 Urinary Metabolites of [^{14}C]Benzoic Acid in the Ferret.

3 female ferrets were dosed 100mg/kg i.p. + 10.9μCi.

Time (h)	Recovery (%)	% dose unchanged	% dose conjugated with	
			glycine	glucuronic acid
0-3	20 (13-24)	0.5 (0.3-0.8)	7 (2-10)	13 (10-14)
0-24	86 (67-96)	1.7 (1.4-1.9)	34 (23-39)	51 (43-55)

Benzoyltaurine was not detected by radiochromatogram scanning and reverse isotope dilution (< 0.1% of dose).

Table 3.12 24h Urinary Metabolites of [^{14}C]Benzoic Acid in the Cat.

1 female and 2 neutered male cats were dosed 100mg/kg i.p. + 8.7μCi.

Recovery (%)	% dose unchanged	% dose conjugated with glycine
70 (45-94)	0	70 (45-94)

Benzoyltaurine and benzoylglucuronide were not detected.

Table 3.13 24h Urinary Metabolites of [^{14}C]Benzoic Acid in the Indian Fruit Bat.

Bats were dosed 100mg/kg i.p. + 11μCi.

Bat No.	Sex	Recovery (%)	% of 24h excretion of ^{14}C as				
			Unchanged acid	Benzoyl-glucuronide	Benzoyl-glutamic acid		Hippuric acid
1	F	69 §	0	88	8	(9)†	0†
1	F	78	3	86	11	-	0†
2	F	92	0	95	5	(5)†	0†
3	M	96	tr.	84	16	(15)†	0†
Mean values:		84	1	88	10	(10)	0

F = female; M = male.

0 means not detected (<0.5% of ^{14}C excreted).

tr. means trace (0.5-1% of the ^{14}C excreted).

† Determined by reverse isotope dilution.

§ Urine contained an unidentified peak.

Table 3.14 24h Urinary Metabolites of [^{14}C]Benzoic Acid in the African Fruit Bat, *Epomops franqueti*.

Bats were dosed 100mg/kg.

Bat No.	Dose of ^{14}C µCi/bat	Recovery (%)	% of 24h excretion of ^{14}C as: unchanged acid †	Benzoyl-glucuronide†	Benzoyl-glutamic acid§	Hippuric acid §	Benzoyl glutamic acid + Hippuric acid †
1	8	38	35	40	10	17	24
2	8	19	49	6	-	44	46
2	102	75 *	29	33	0.5	22	24
3	8	33	26	37	9	31	37
4	102	100	8	27	26	40	66
5	102	59	8	45	10	31	48
Mean values:		54	26	32	11	31	41

† Measured by radiochromatogram scanning.

§ Measured by r.i.d.

* Urine contained an unidentified peak at R_F 0.02 (14% of ^{14}C).

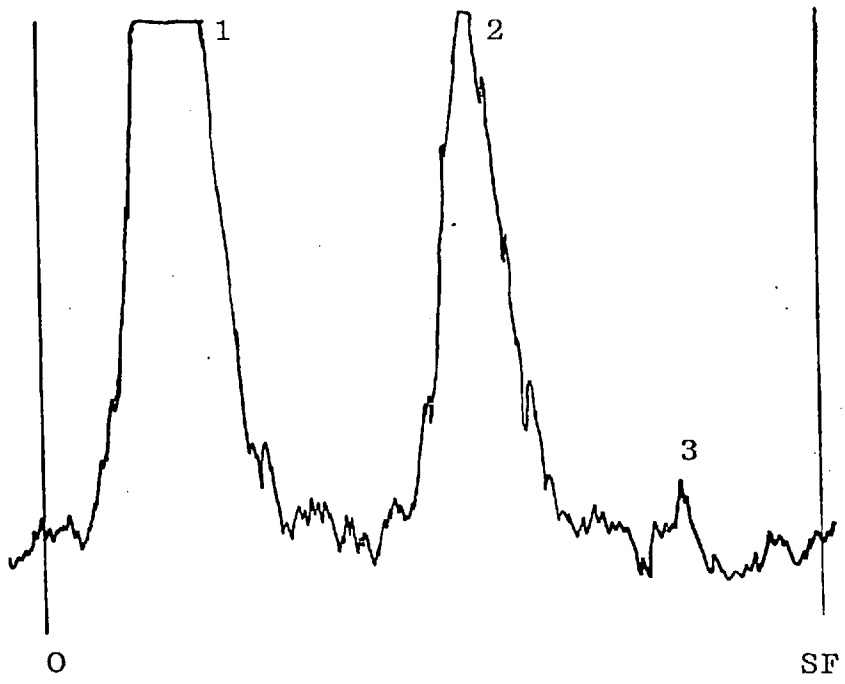
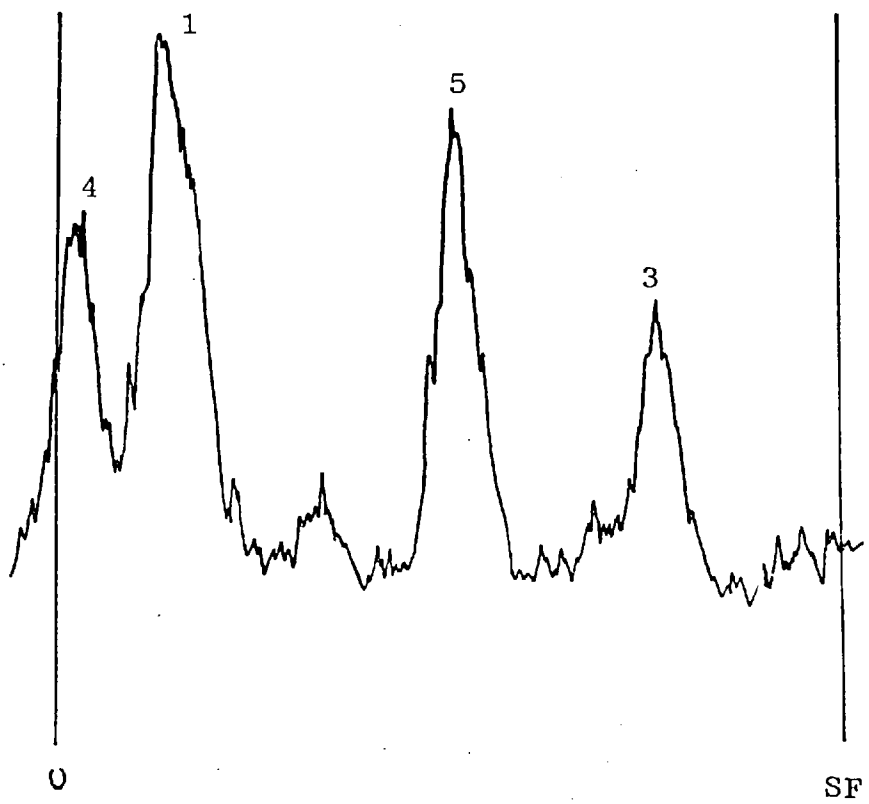


Fig.3.6 Radiochromatogram Scan of Urine from an Indian Fruit Bat given [^{14}C]Benzoic Acid.

(See page 150 for Key).

Fig.3.7 Radiochromatogram Scan of Urine from a Franquet's Fruit Bat given [^{14}C]Benzoic Acid.



Key to Figs.3.6 and 3.7.

1 = benzoylglucuronide

2 = benzoylglutamic acid

3 = benzoic acid

4 = unidentified conjugate

5 = benzoylglutamic acid + hippuric acid.

Metabolism of 4-Nitrobenzoic Acid in the Ferret and Rat.

The metabolism of [^{14}C]4-nitrobenzoic acid in the ferret is shown in Table 3.15. 74% of the dose was recovered in the 24h urines and of this 38% was excreted as 4-nitrohippuric acid and 29% as 4-nitrobenzoylglucuronide. Two minor peaks were also found, which corresponded to 4-acetylaminobenzoic acid and 4-acetylaminohippuric acid on t.l.c. (see Fig.3.8). These reduced and acetylated metabolites accounted for 3.2% of the dose, whilst Bratton and Marshall assay gave a figure of 5.8% of the dose reduced in these urines (see later).

The metabolism of [^{14}C]4-nitrobenzoic acid in the rat is shown in Table 3.16. In contrast to the ferret, which excreted only 4% of the dose unchanged in 24h, the rat excreted 38% of the dose unmetabolised in the 24h urine. The major metabolite found on t.l.c. was 4-acetylaminobenzoic acid (44% of the dose), and only small amounts of this compound were conjugated through the carboxyl group (7% of the dose). A total of 47% of the dose appeared as reduced metabolites, which is in fair agreement with the colorimetric data described later which gave the figure of 38% of the dose reduced. A radiochromatogram scan is shown in Fig.3.9. This

Table 3.15 24h Urinary Metabolites of [^{14}C]4-Nitrobenzoic Acid in the Ferret.

3 female ferrets were dosed 100mg/kg i.p. + 6.4μCi.

Recovery (%)	% dose unchanged	% dose as				Total reduced (%) §
		4-nitrohippuric acid	4-nitrobenzoyl-glucuronide	4-acetylamino-benzoic acid	4-acetylamino-hippuric acid	
74 (70-79)	3.9 (3.8-4.0)	38 (33-45)	29 (24-34)	1.6 (0-4)	1.7 (1-2)	3.2 (2-5)

§ Bratton and Marshall assay gave a figure of 5.8% total reduction (see later).

Table 3.16 24h Urinary Metabolites of [^{14}C]4-Nitrobenzoic Acid in the Rat.

4 female rats were dosed 100mg/kg i.p. + 1.6μCi and their urines were pooled for analysis.

Recovery (%)	% dose unchanged	% dose as			Total reduced (%) §
		4-nitrohippuric acid	4-aminohippuric acid	4-acetylamino-benzoic acid	
89	38	4	3	44	47

§ Bratton and Marshall assay gave a figure of 38% total reduction (see later).

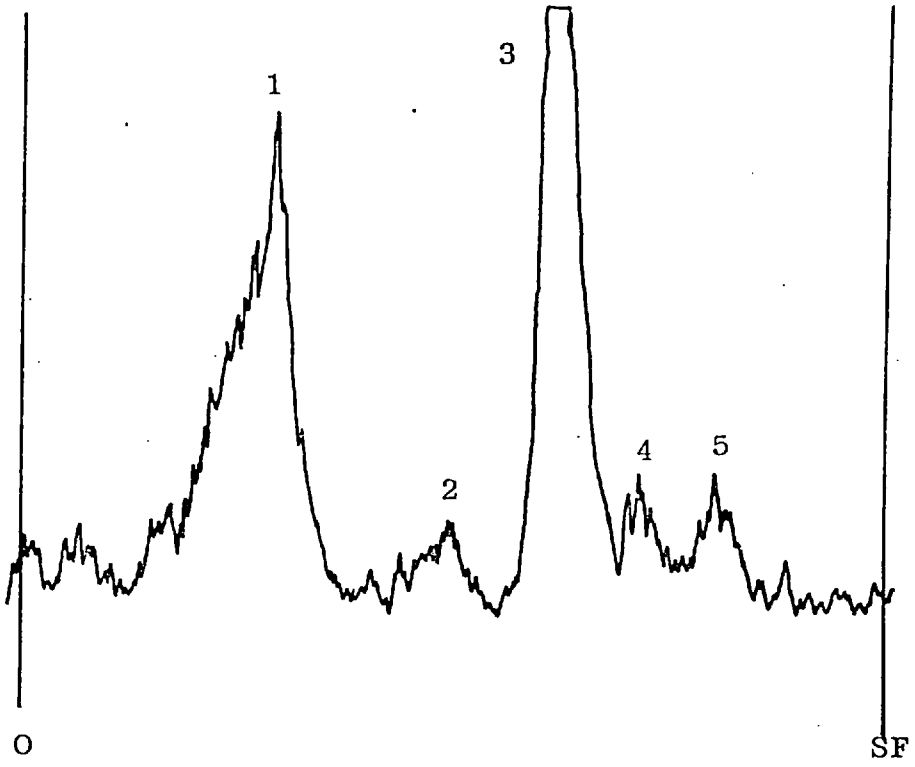
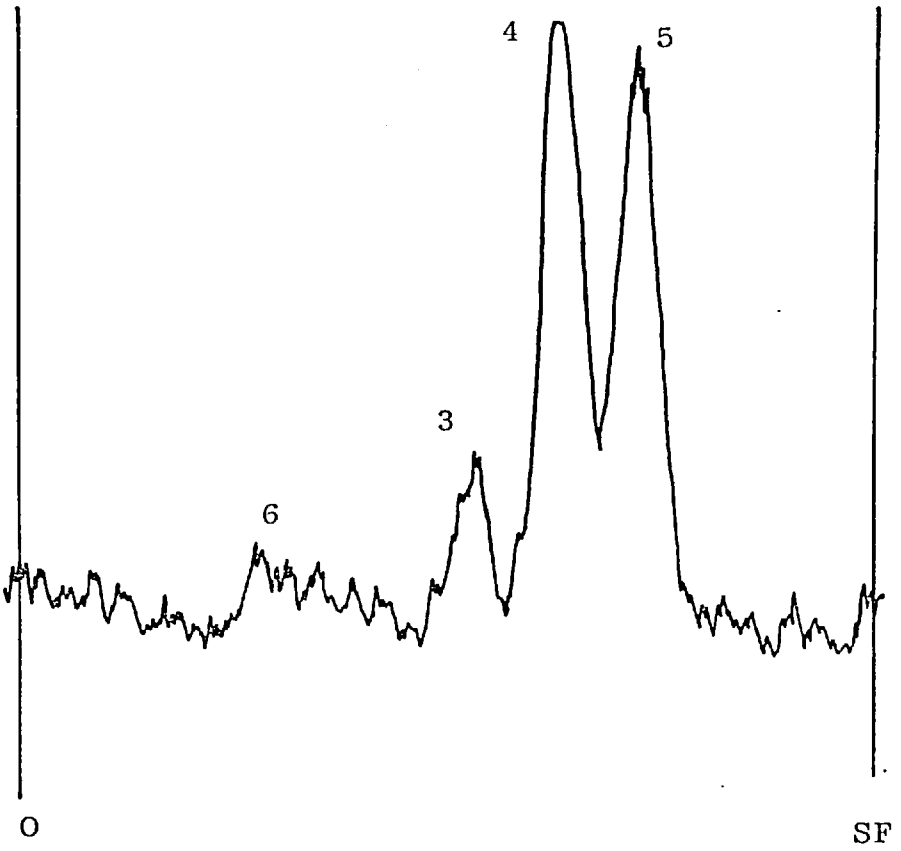


Fig.3.8 Radiochromatogram Scan of Urine from a Ferret given [^{14}C] 4-Nitrobenzoic Acid.

(See page 153 for Key).

Fig.3.9 Radiochromatogram Scan of Urine from Rats given [^{14}C] 4-Nitrobenzoic Acid.



demonstrates a marked species difference in the metabolism of 4-nitrobenzoic acid between the ferret and the rat which has not previously been noticed.

Key to Figs.3.8 and 3.9.

1 = 4-nitrobenzoylglucuronide

2 = 4-acetylaminohippuric acid

3 = 4-nitrohippuric acid

4 = 4-acetylaminobenzoic acid

5 = 4-nitrobenzoic acid

6 = 4-aminohippuric acid.

Metabolism of Indol-3-ylacetic Acid in the Ferret.

The metabolism of [^{14}C]indol-3-ylacetic acid in the ferret is shown in Table 3.17. This compound is conjugated in the ferret with both glycine (18% of the dose in 24h) and taurine (34%). The metabolism of indol-3-ylacetic acid in the ferret is like that of the three phenylacetic acids described previously, in that it is conjugated only with the amino acids glycine and taurine and not with glucuronic acid.

Metabolism of 1-Naphthylacetic Acid in the Ferret and Dog.

The metabolism of [^{14}C]1-naphthylacetic acid in the ferret and dog is shown in Table 3.18. Whereas the ferret excreted mainly the taurine conjugate (49% of the dose) and some glucuronide (20%), the dog made mainly the glycine conjugate (57%) with very little glucuronide (6% in one animal). Dixon *et al.* (1974) showed that 1-naphthylacetic acid was conjugated extensively with glucuronic acid in man, three

species of monkey, the rabbit and the Indian fruit bat. In the bushbaby and the cat however, this acid was conjugated mainly with glycine and taurine. The two species of carnivore studied here are qualitatively similar to the bushbaby in that they make glycine, taurine and glucuronic acid conjugates, but differ quantitatively to other species studied. The ferret is unusual because it excretes 1-naphthylacetyltaurine as the major metabolite (49% of the dose, 63% of the 24h urinary ^{14}C).

Table 3.17 Urinary Metabolites of [^{14}C]Indol-3-ylacetic Acid in the Ferret.

3 female ferrets were dosed 100mg/kg i.p. + 5μCi.

Duration of experiment (h)	Recovery (%)	% dose unchanged	% dose conjugated with	
			glycine	taurine
3	35 (22,47)	18 (8,27)	4 (3,6)	13 (8,17)
24	82 (64-97)	30 (22-37)	18 (8-28)	34 (24-41)
48	84 (66-100)	30 (22-37)	19 (8-29)	35 (24-41)

Indol-3-ylacetylglutamine and indol-3-ylglucuronic acid were not detected.

Table 3.18 Urinary Metabolites of [^{14}C]1-Naphthylacetic Acid in the Ferret and Dog.

3 female ferrets were dosed 100mg/kg i.p. + 10μCi. 2 female dogs were dosed 100mg/kg p.o. + 9.4μCi.

Species	Duration of experiment (days)	Recovery (%)	% dose unchanged	% dose conjugated with		
				glycine	taurine	glucuronic acid
Ferret	1	50 (40-61)	2 (2-3)	3 (2-3)	32 (25-42)	13 (11-15)
	2	78 (66-88)	3 (3-4)	5 (3-7)	49 (42-59)	20 (19-22)
Dog	1	†76 (76,76)	3 (1,5)	57 (71,43)	11 (4,19)	3 (0,6)

† Peak appeared in one urine corresponding to 1-naphthylacetylglutamine (3.4% of the dose) by t.l.c.

Metabolism of 2-Naphthylacetic Acid in the Ferret.

The metabolism of [^{14}C]2-naphthylacetic acid in the ferret is shown in Table 3.19. A very low recovery of radioactivity (21% in 24h) was obtained, which may be related to impaired hepatic or renal function since two animals died during the experiment and one had to be destroyed at 24h. The principal metabolite found was the taurine conjugate (15% of the dose, 71% of the urinary ^{14}C). Some glucuronic acid and glycine conjugates were also detected and thus the ferret metabolised this acid in a similar way to 1-naphthylacetic acid. The vast difference in toxicity of these two acids is of great interest, since they appear in urine similarly conjugated.

Table 3.19 24h Urinary Metabolites of [^{14}C]2-Naphthylacetic Acid in the Ferret.

3 female ferrets were dosed 100mg/kg i.p. + 9.4μCi.

Recovery		% dose conjugated with		
(%)	unchanged	glycine	taurine	glucuronic acid
† 21	0.7	1.5	15	3
(17-24)	(0.3-1.5)	(1.1-1.8)	(12-18)	(2-6)

† Two animals died during the experiment and one was destroyed at 24h. *Post mortem* examination showed a haemoperitoneum in each animal.

Metabolism of Diphenylacetic Acid in the Ferret.

The metabolism of [^{14}C]diphenylacetic acid in the ferret is shown in Table 3.20. Only the glucuronide (32% of the dose in 48h) was detected with this compound. A total of 70% of the ^{14}C was excreted in urine and faeces in 4 days. The molecular weight of diphenylacetylglucuronide is 388 and thus this metabolite may be excreted in the bile and emerge in the faeces. The factors affecting the biliary excretion of organic compounds are discussed by Smith (1973). The formation of solely the glucuronide of diphenylacetic acid is in keeping with observations in man, dog and rabbit reported in the older literature (Miriam *et al.*, 1927*a*, *b*).

Table 3.20 Urinary Metabolites of [^{14}C]Diphenylacetic Acid in the Ferret.

3 female ferrets were dosed 100mg/kg i.p. + 5.2μCi.

Duration of experiment (days)	Recovery (%)	% dose unchanged	% dose conjugated with glucuronic acid
1	38 (19-50)	14 (6-20)	24 (12-34)
2	†48 (26-63)	16 (9-21)	32 (17-44)
4	63 (55-71)	-	-

† 7% (2-14) radioactivity was detected in the 0-4day faeces.

- Means not determined.

Diphenylacetylglycine, diphenylacetyltaurine and diphenylacetylglutamine were not detected.

Metabolism of (±)-2-Phenylpropionic Acid in the Ferret.

The metabolism of [^{14}C]2-phenylpropionic acid (hydratropic acid) in the ferret is shown in Table 3.21. The ferret conjugated this acid with glycine (32% of the dose), taurine (38%) and glucuronic acid (6%) and in this respect 2-phenylpropionic acid is metabolised much like the two naphthylacetic acids in this species. 2-Phenylpropionylglycine has been reported in the dog (Kay & Raper, 1922), and Robinson *et al.* (1955) showed that when the (+)-, (-)- or (±)-2-phenylpropionic acids were given to rabbits they were largely excreted as the glucuronides. Whether any partial resolution of the racemate occurred in the ferret was not determined.

Table 3.21 24h Urinary Metabolites of [^{14}C](±)-2-Phenylpropionic Acid in the Ferret.

3 female ferrets were dosed 100mg/kg i.p. + 13μCi.

Recovery (%)	% dose unchanged	% dose conjugated with		
		glycine§	taurine§	glucuronic acid
77	1.3	32	38	6
(73-81)	(0.6-2.3)	(25-38)	(34-41)	(4-7)

§ Both glycine and taurine conjugates identified as described earlier by hydrolysis and determination of the liberated amino acids.

Reduction and Acetylation of 4-Nitrophenylacetic Acid by Man, Dog, Cat, Ferret, Pigeon and Hen.

[^{14}C]4-nitrophenylacetic acid was administered to various species to study the metabolic pattern arising from conjugation of the carboxyl group. However, a further change which aromatic nitro acids may undergo *in vivo* is reduction of the nitro group. Cohn (1893, 1894) was the first to observe this, isolating 3-acetylaminobenzoic acid as a metabolite of 3-nitrobenzaldehyde and 3-nitrobenzoic acid in the rabbit; he also isolated 4-acetylaminobenzoic acid as a metabolite of 4-nitrobenzaldehyde.Sherwin & Hynes (1921) did not observe any reduction of nitrobenzaldehydes in man. Kohl & Flynn (1941) studied the reduction of the nitrobenzoic acids by rat liver *in vitro*. They found that for the 3- and 4-nitrobenzoic acids, about 30% of the dose was reduced in 7h; but for 2-nitrobenzoic acid only 1-2% of the dose was reduced. Bray *et al.* (1949) showed that 11-21% of the dose of the nitrobenzoic acids was reduced in the rabbit and that acetylation occurred for the 3- and 4-isomers only. Fouts & Brodie (1957) studied the reduction of chloramphenicol, 4-nitrobenzoic acid and a variety of other aromatic nitro compounds to the corresponding amines *in vitro*. In rabbits they found a nitroreductase system in a number of tissues with the liver being most active. Kidney had about one-half the activity of liver; lung and heart had a small but measurable activity; brain, muscle and blood had little or none. The reduction of 4-nitrobenzoic acid was compared in liver homogenates of various animal species. The livers of mice and guinea-pigs showed the highest activity. These were about three times as active as those of rabbits, while those of rats and dogs had relatively little activity.

No reduced metabolites of 4-nitrophenylacetic acid have been reported. James *et al.* (1972*a*) found that this acid was eliminated almost totally unchanged in man and rhesus monkey with a small unknown peak (about 5% of the dose) which yielded 4-nitrophenylacetic acid on acid hydrolysis. In the rat 61% of the dose was conjugated with glycine and 28% of the dose eliminated as unchanged acid.

The extent of nitro-reduction and subsequent acetylation of [^{14}C]4-nitrophenylacetic acid in various species has been determined using the colorimetric method of Bratton & Marshall (1939) and the results are shown in Table 3.22. In general, the colorimetric results are in fair agreement with data obtained by radiochromatography. In the case of the cat, pigeon and hen, radiochromatography gave lower values than colorimetry for the extent of reduction, but in each case a polar unidentified ^{14}C peak was found which could account for the discrepancy and thus was probably a reduced metabolite. No reduction of 4-nitrophenylacetic acid was found in man at a dose level of 0.5mg/kg p.o. The dog was found to reduce 5% of the dose in 48h, of which none was acetylated. This was proven by analysing the optical densities for free and total amine (see p. 121) by Student's t test. No significant difference was found ($P>0.1$) and thus no acetylation could be inferred. This observation is consistent with previous findings that the dog does not acetylate aromatic amines (see Williams, 1967). The cat was found to reduce 25% of the dose in 48h, which was mainly acetylated (22%). Reduced metabolites were seen on radiochromatogram scanning of the urine (see Table 3.10). The ferret however, excreted only 6% of the dose as reduced metabolites, of which approximately half was acetylated ($P<0.001$).

The two species of bird studied showed a distinct species difference. Whereas the pigeon excreted 4% of the dose as reduced metabolites, the domestic hen reduced 14% of the dose, of which 11% was acetylated. The conjugation of 4-nitrophenylacetic acid in these two species of bird has been reported elsewhere (Idle *et al.*, 1976). In conclusion, both the cat and hen seem to be efficient at reducing this acid, the cat excreting mainly acetylamino metabolites and the hen mainly free amino metabolites.

Reduction and Acetylation of 4-Nitrobenzoic Acid by Dog, Ferret and Rat.

The reduction of 4-nitrobenzoic acid in the dog, ferret and rat is given in Table 3.23. Both species of carnivore reduced this acid to a similar extent in 24h, and again no statistically significant extent of acetylation could be demonstrated in the dog. The rat showed a very high extent of reduction, the figures being 38% of the dose by colorimetric determination and 47% by radiochromatography. This result is in contrast to the findings of Fouts & Brodie (1957) that dog and rat liver homogenates both had very little nitrobenzoic acid reductase activity *in vitro*. It is apparent that in this case, the *in vivo* findings are vastly different to the *in vitro* data, the rat producing a large amount of reduced urinary metabolites and the dog relatively little.

Table 3.22 Nitro-reduction and Acetylation of 4-Nitrophenylacetic Acid in Various Species.

Human volunteers were given 0.5mg/kg p.o. and all other animals were dosed 100mg/kg i.p.

Species	No.	Sex	Duration of experiment (days)	^{14}C administered (μCi)	^{14}C recovered in urine (%)	% dose in urine as 4-aminophenyl-acetic acid derivatives	% dose in urine as 4-acetylamino-phenylacetic acid derivatives	Total reduced (% dose) C.	Total reduced (% dose) R.S.
Man	3	M	0 - 1	0.2	100	0	0	0	-
Dog	3	F	0 - 1	9.3	67	2.4	0	2.4	-
			1 - 2		10	2.7	0	2.7 (5.1)¶	-
Cat	3	F	0 - 1	9.9	56	2.1	12.8	14.9	11†
			1 - 2		26	1.2	9.0	10.2 (25.1)	-
Ferret	3	F	0 - 1	0.3	40	0.9	1.5	2.4	-
			1 - 2		22	1.3	0.7	2.0 (4.4)	-
			2 - 3		14	0.7	1.1	1.8 (6.2)	-
Pigeon*	4	2F,2M	0 - 1	8.7	75	1.8	1.8	3.6	1.0§
Hen *	3	F	0 - 1	8.9	54	10.6	3.4	14.2	4.3 ††

Only mean values are given. Ranges have been omitted for the sake of clarity. /over.

Table 3.22 Contd.

Key

M = male, F = female.

C = estimated by colorimetry (Bratton & Marshall).

R.S. = estimated by radiochromatogram scanning.

0 means not detected (<0.1% of the dose). - means not determined.

* The conjugation of 4-nitrophenylacetic acid in these two species has been described elsewhere (Idle *et al.*, 1976).

¶ Figures in parentheses are cumulative values.

† See Table 3.10 for details of conjugation.

§ Radiochromatography of pigeon excreta showed an unidentified peak (R_F 0.00 in system C) which did not correspond to any of the possible metabolites given in Table 2.6 and accounted for 3.4% of the dose.

†† As for § , but accounting for 9.6% of the dose.

Table 3.23 Nitro-reduction and Acetylation of 4-Nitrobenzoic Acid by Dog, Ferret and Rat.

All animals were dosed 100mg/kg i.p.

Species	No.	Sex	Duration of experiment (days)	^{14}C administered (μCi)	^{14}C recovered in urine (%)	% dose in urine as 4-amino-benzoic acid derivatives	% dose in urine as 4-acetylamino-benzoic acid derivatives	Total reduced (% dose) C	Total reduced (% dose) R.S†
Dog	2	F	0-1	11.3	93	6.0	0 §	6.0	-
Ferret	3	F	0-1	6.4	74	3.9	1.9 §	5.8	3.2
			1-2		5	2.0	1.4	3.4 (9.2)	-
Rat	4	F	0-1	1.6	89	14.9	23.2	38.1	47

§ Difference between free and total amine shown by Student's t test (ferret, $P<0.001$; dog, $P>0.1$)

† See Tables 3.15 and 3.16 for details of conjugation.

See table 3.22 for other relevant footnotes.

CHAPTER FOUR

Compilation of Results and Concluding Remarks.

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The Metabolism of some Aromatic Acids in the Ferret.

The metabolism of ten aromatic acids in the ferret has been summarised in Table 4.1. A dose of 100mg/kg i.p. was used and not a molar equivalent dose of each acid, since the molecular weights of the acids used fell into a fairly narrow range [164 ± 25 (±15%) standard deviations]. The effect of dose level has also been studied with phenylacetic acid in the ferret (see Table 3.3). Thus it was felt that doses of 100mg/kg would be valid when comparing general patterns of metabolism of aromatic acids. Table 4.1 clearly shows that these acids can be metabolised in two distinct ways with a graded response in between. Phenylacetic, 4-chlorophenylacetic, 4-nitrophenylacetic and indol-3-ylacetic acids were conjugated only with amino acids, including taurine, and not with glucuronic acid, whilst diphenylacetic acid in the ferret was conjugated only with glucuronic acid. However, 1- and 2-naphthylacetic, 2-phenylpropionic, benzoic and 4-nitrobenzoic acids were all conjugated with both amino acids and glucuronic acid. The two benzoic acids studied though, did not make a taurine conjugate. Presumably, the benzoyl-CoA derivatives, unlike the phenylacetyl-CoA derivatives, are not good substrates for the taurine N-acyltransferase enzyme, and once activated in this way the intermediate reacts with glycine in preference. Taurine conjugation was found as a metabolic reaction of seven of the acids studied, in five of which it was the major biotransformation. All acids except diphenylacetic acid formed a glycine conjugate and this was the dominant reaction of three of these acids.

Does the data derived from this study suggest a structure-activity relationship? The results in the ferret seem to

Table 4.1 Conjugation of some Aromatic Acids in the Ferret at a Dose of 100mg/kg i.p.

Compound given	^{14}C excreted (% of dose)	Time (days)	% of ^{14}C excreted unchanged	% of ^{14}C excreted conjugated with		
				glycine	taurine	glucuronic acid
a) Benzoic acids						
Benzoic	86	1	1	40	0	59
4-Nitrobenzoic *	74	1	5	51	0	38
b) Arylacetic acids						
Phenyl †	68	1	3	63	21	0
4-Chlorophenyl §	87	1	54	30	9	0
4-Nitrophenyl	56	4	25	12	63	0
Indol-3-yl	82	1	37	22	41	0
1-Naphthyl	78	2	4	6	63	26
2-Naphthyl	21	1	5	10	71	14
Diphenyl	48	2	33	0	0	67
c) Arylpropionic acids						
(±)-2-Phenyl	77	1	2	42	49	8

/over.

Table 4.1 Contd.

Key

* 4-Acetylaminobenzoic acid (3% of ^{14}C excreted) and 4-acetylaminohippuric acid (3%) were also found.

† An unknown peak amounted to 13% of the ^{14}C (partially accounted for as the glutamine conjugate).

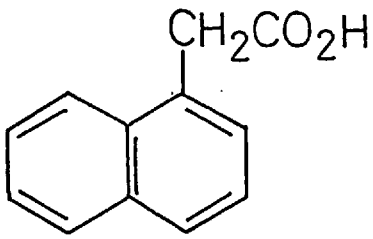
§ An unknown peak amounted to 7% of the ^{14}C.

conform to the hypothesis proposed initially that the primary mode of conjugation of aromatic acids is with glycine, glucuronic acid conjugation occurring when the carboxyl centre becomes sterically shielded to nucleophilic attack whether hindering formation of the AMP(adenylate) intermediate or the subsequent CoA derivative. The three phenylacetic acids and indol-3-ylacetic acid have their carboxyl centres remote enough from any sterically-demanding moiety to allow amino acid conjugation to ensue. Diphenylacetic acid, as shown in Fig.1.13, has a sterically hindered carboxyl group (*cf* 4-phenylphenylacetic acid; Fig.1.13) and thus forms only the glucuronide. 1- and 2-naphthylacetic acids both possess aromatic substituents which render the molecule more sterically demanding than phenylacetic acid (see Fig.4.1). Therefore, in accordance with the hypothesis, more glucuronide is formed with 1-naphthylacetic acid (a 2,3-disubstituted phenylacetic acid) and 2-naphthylacetic acid (3,4-disubstituted) than with phenylacetic acid.

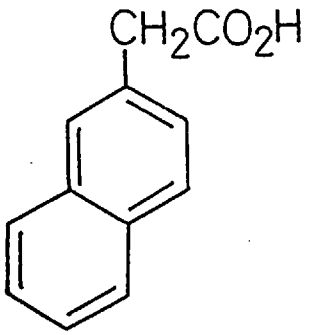
A similar rationalisation of the data can be drawn up for diphenylacetic acid and 2-phenylpropionic acid. As shown earlier, α-substitution may have a radical effect upon the pattern of conjugation of arylacetic acids and therefore the size of the substituent, *i.e.* its steric demand, would be important in determining the magnitude of such an effect. This is demonstrated in Fig.4.2. As the size of the α-substituent increases from hydrogen to phenyl, the degree of glucuronidation increases.

The observed conjugation of the two benzoic acids is more difficult to explain. In general, benzoic acid forms a glycine conjugate in most species to which it is administered and this is in keeping with the general hypothesis proposed

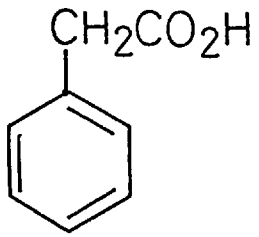
Fig.4.1 Steric Demand of 1- and 2-Naphthylacetic Acids as Compared with Phenylacetic Acid.



1-naphthylacetic acid (26%)



2-naphthylacetic acid (14%)

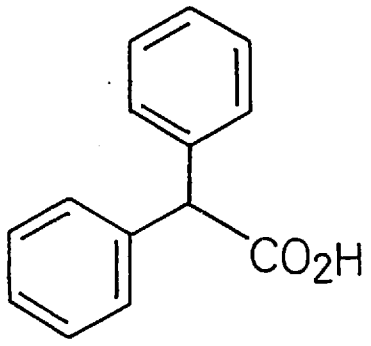


phenylacetic acid (0%)

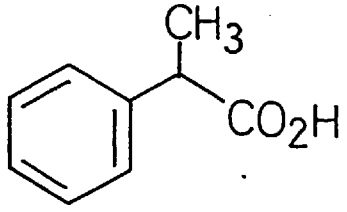
decreasing steric hindrance of carboxyl group; decreasing glucuronide formation in the ferret.

Figures in parentheses are % of 24h urinary ^{14}C as glucuronide in the ferret at 100mg/kg i.p. dose.

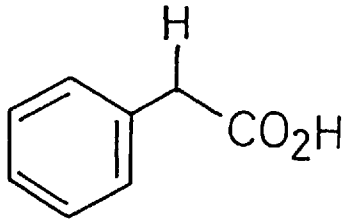
Fig.4.2 Steric Demand of Diphenylacetic Acid and 2-Phenylpropionic Acid as Compared with Phenylacetic Acid.



diphenylacetic acid (67%)



2-phenylpropionic acid (8%)



phenylacetic acid (0%)

decreasing steric hindrance of carboxyl group; decreasing glucuronide formation in the ferret.

Figures in parentheses are % of 24h urinary ^{14}C as glucuronide in the ferret at 100mg/kg i.p. dose.

here. However, it is known that in the dog (see Williams, 1959) and in the ferret (Bridges *et al.*, 1970), increasing the dose of benzoic acid evokes a greater production of the glucuronide at the expense of hippuric acid and it is believed to be the supply of glycine which is rate limiting in such species as the dog. However, it is still not clear whether the availability of the amino acid is indeed the determining factor in a species such as the dog or ferret. The dog is able to mobilise sufficient of the amino acid to conjugate with glycine 100% of a dose of phenylacetic acid given at 100mg/kg i.p. (see Table 3.4). Nor is there any real correlation between the steady state hepatic and renal taurine levels as shown in Table 1.17, and the extent of conjugation of phenylacetic acid with taurine in various species (see James *et al.*, 1972*a*). The mobilisation of the amino acid may be rate limiting towards one substrate but not another.

The Metabolism of some Aromatic Acids in the Cat and Dog.

The conjugation of some aromatic acids in the cat is summarised in Table 4.2. Glucuronide was not found for any acid and the three phenylacetic acids were found to form glycine and taurine conjugates. The data for 1-naphthylacetic acid (Dixon *et al.*, 1974) and indol-3-ylacetic acid (Bridges *et al.*, 1974) has been added for comparison.

The conjugation of some aromatic acids in the dog is summarised in Table 4.3. The data for indol-3-ylacetic acid (Bridges *et al.*, 1974) has been added for comparison. Except for 4-nitrophenylacetic acid, which was largely excreted unchanged in the dog, the acids given were all mainly conjugated with glycine, the taurine conjugate also being found for 4-chlorophenylacetic acid (20% of the ^{14}C), 4-nitro-

Table 4.2 Conjugation of some Aromatic Acids in the Cat at a Dose of 100mg/kg i.p.

Compound given	^{14}C excreted (% of dose)	Time (days)	% of ^{14}C excreted unchanged	% of ^{14}C conjugated with		
				glycine	taurine	glucuronic acid
a) Benzoic acids						
Benzoic	70	1	0	100	0	0
b) Arylacetic acids						
Phenyl	100	1	9	90	1	0
4-Chlorophenyl*	100	2	2	92	2	0
4-Nitrophenyl ¶	56	1	39	4	16	0
1-Naphthyl †	n.g.	1	2	59	39	0
Indol-3-yl §	93	2	24	75	-	0

* Urine contained an unidentified metabolite corresponding to 4% of the ^{14}C.

¶ 4-Acetylaminophenylacetic acid amounted to 20% of the ^{14}C and an unidentified metabolite (20%) was also found.

† Taken from Dixon *et al.* (1974)

§ Taken from Bridges *et al.* (1974).

\- means not looked for.

0 means not detected.

n.g. means not given in the literature.

Table 4.3 Conjugation of some Aromatic Acids in the Dog at a Dose of 100mg/kg.

Arylacetic acid given	Route	% ^{14}C excreted	Time (days)	% of ^{14}C excreted unchanged	% of ^{14}C conjugated with		
					glycine	taurine	glucuronic acid
Phenyl	i.p.	100	1	0	100	0	0
4-Chlorophenyl *	i.p.	98	1	2	77	20	0
4-Nitrophenyl	i.p.	76	1	80	0	20	0
1-Naphthyl ¶	p.o.	76	1	4	75	14	4
Indol-3-yl †	i.p.	83	2	30	70	-	0

* Urine contained unidentified metabolite (2% of ^{14}C).

¶ Urine contained unidentified metabolite (4% of ^{14}C).

† Taken from Bridges *et al.* (1974).

See Table 4.2 for other relevant footnotes.

phenylacetic acid (20%) and 1-naphthylacetic acid (14%).

Glycine Conjugation in the Order *Chiroptera* and its Importance to Pharmacozootaxonomy and Drug Metabolism.

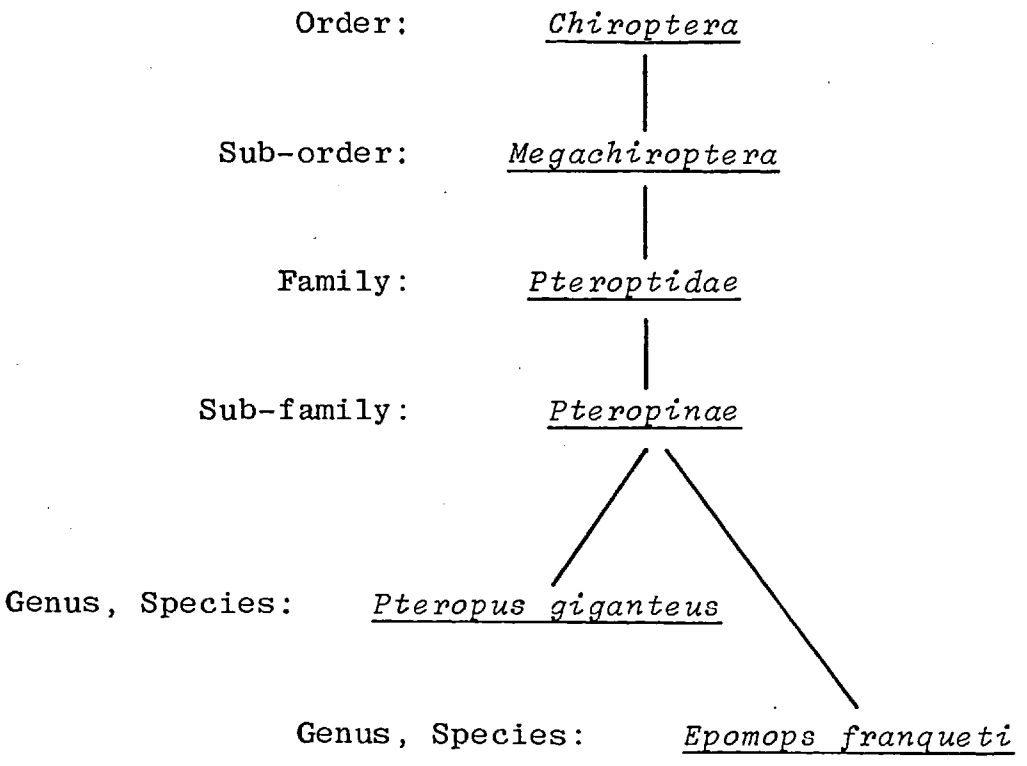
Pharmacozootaxonomy is a word derived by Williams (1976) to describe studies in foreign compound metabolism which can assist in the classification of species. Since the original study by Bridges *et al.* (1970), the Indian fruit bat (*Pteropus giganteus*) has appeared to be unique in that it does not synthesize hippuric acid from administered benzoic acid. Work in this laboratory subsequently confirmed these findings (Bababunmi *et al.*, 1973; Ette *et al.*, 1974) and it has now been demonstrated (see Table 3.13) that this bat makes benzoyl-L-(+)-glutamic acid (5-15% of the urinary ^{14}C), a novel metabolite in vertebrates. It was of immediate interest to study the fate of [^{14}C]benzoic acid in a closely related species of fruit bat to see whether the absence of hippuric acid synthesis was peculiar to *Pteropus giganteus*. The metabolism of [^{14}C]benzoic acid was therefore studied in the African bat, *Epomops franqueti* and this bat was found to make both hippuric acid and benzoylglutamic acid (see Table 3.14). This represents a marked species difference between two species whose close taxonomic relationship is shown in Fig.4.3. This shows that, as far as we are aware, the "defect" in hippuric acid synthesis occurs at the level of the Genus and is not peculiar to any Sub-order, Family or Sub-family of the *Chiroptera*. More work must be done in this field to screen the hippuric acid and benzoylglutamic acid forming capacity of other species of bat, both closely and distantly related to *Pteropus giganteus* to see if there is a true parallel between a taxonomic (based on anatomy and morphology) and a pharmaco-

zootaxonomic (based on drug metabolism) classification of bats. The work should also be extended to other aromatic acids, both substituted benzoic and arylacetic acids, to discover if the "defect" is truly one of hippuric acid formation or can be extended more generally to glycine conjugation of a group of substrates.

A second important principle emerges from the study of aromatic acid transformations in bats; that of *substrate directed metabolism* and *species directed metabolism*. When a xenobiotic enters the animal body it may be metabolised by a number of routes. The major factor governing the possible directions of metabolism is the chemical nature of the compound itself, since if it did not contain a nitrogen atom, for example, it could not undergo *N*-oxidation. Conversely, there are various properties of the compound which will preclude metabolism along certain pathways. For example, extremely polar acidic compounds such as saccharin do not appear to undergo any biotransformations (Ball *et al.*, 1976). Similarly according to the *steric hypothesis* proposed here, phenylacetic acid is conjugated with amino acids and not glucuronic acid in all species studied [the phenylacetylglucuronide described by James *et al.* (1972*a*) could not be demonstrated in this work at 100mg/kg]. This illustrates the idea of *substrate directed metabolism*. The most poignant examples emerge from the study of species "defects". The Indian fruit bat does not make hippuric acid and Dixon *et al.* (1974) also showed that the one bat studied did not conjugate 1-naphthylacetic acid with glycine. However, the work of Ette *et al.* (1974) clearly showed the formation of the glycine conjugate of phenylacetic acid in this species of bat. Thus the nature

of the compound prevails and *substrate directed metabolism* results. *Species directed metabolism* may be the general rule, as for glucuronide formation in the cat, but there are certain compounds such as phenolphthalein which are conjugated with glucuronic acid in this species (Capel *et al.*, 1974). Therefore, a clear definition of both substrate and species is required.

Fig.4.3 Taxonomic Relationship between *Pteropus giganteus* and *Epomops franqueti.*



Classification according to Simpson (1945).

Substrate Directed Metabolism, the Steric Hypothesis and and Concluding Remarks.

The steric hypothesis gives a good predictive basis for the direction of conjugation of aromatic acids which cannot otherwise be obtained from such properties as pKa or log P. In general, the unhindered acetic, propionic and oxyacetic unsubstituted sidechains are conjugated with amino acids in all species and thus demonstrate *substrate directed metabolism*. The hindered acids, especially benzoic acids with one or two large aromatic substituents or arylacetic and arylpropionic acids with large α-substituents (ethyl or phenyl) are alternatively conjugated with glucuronic acid. There is a graded response in between where smaller ring- and sidechain-substituted acids (such as 2-phenylpropionic acid and the naphthylacetic acids) are conjugated with both amino acids and glucuronic acid. The nature of the amino acid used for conjugation may also be substrate determined. The older dogma of drug metabolism, for example that glutamine conjugation occurs only in primates, may indeed need some modification. Phenylacetylglutamine has now been demonstrated in the ferret and ^{14}C peaks corresponding to the glutamine conjugates of many of the aromatic acids studied here have been found in such species as the cat, dog and hamster (see Chapter 3). There is also data to suggest that this is so in the rat and the mouse (J.R.Idle, P.Millburn, R.T.Williams & G.Zini, unpublished work). Consequently, glutamine conjugation may be ubiquitous throughout the species, as is taurine conjugation, and thus substrate directed. It is of great interest to know whether such rare reactions as ornithine conjugation in

Galliforms and glutamic acid conjugation in fruit bats are truly species directed metabolic pathways or are of wider distribution among the phyla.

APPENDIX 1

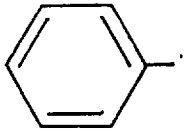
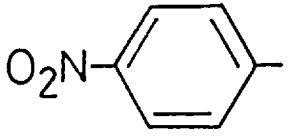
INFRA-RED SPECTRA

Contents

Infra-Red Spectra of some Taurine Conjugates.

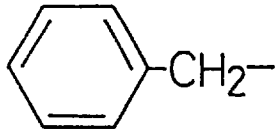
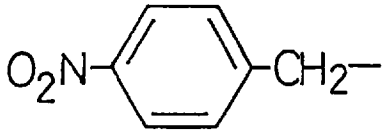
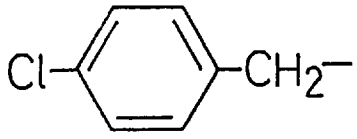
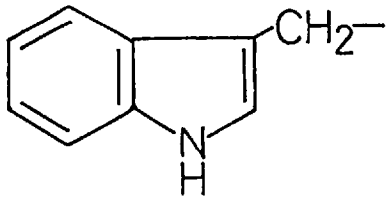
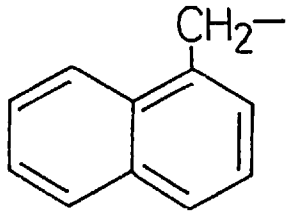
I.R. spectra were measured in paraffin oil (Nujol) mulls using a Perkin-Elmer Spectrophotometer model 137B.

$RCONHCH_2CH_2SO_3H$

R	N-H stretch near 3270cm-1	C=O stretch near 1640cm-1	"Amide II Band" 1570-1515cm-1	SO_3 absorptions 1260-1150 cm-1	SO_3 absorptions 1080-1010 cm-1
CH_3-	3350 3600	1650	1570	1200 1170	1060
CH_3CH_2-	3300	1625	1515	1210 1175	1035
$CH_3CH_2CH_2-$	3300	1625	1515	1210 1175	1035
	3500 3600	1660	1550	1200 1175	1065
	3500 3600	1650	1540	1205	1055

/over.

Infra-Red Spectra of some Taurine Conjugates. Contd.

	3300 3400	1705	1575	1100	1000 1025
	3300 3400	1700	1525	1100	990
	3350	1690	1525	1115 1080	995
	3500 3600	1670	1540	1210	1065
	3400 3600	1650	1570	1210	1040 1060

APPENDIX 2

MASS SPECTRA

Contents

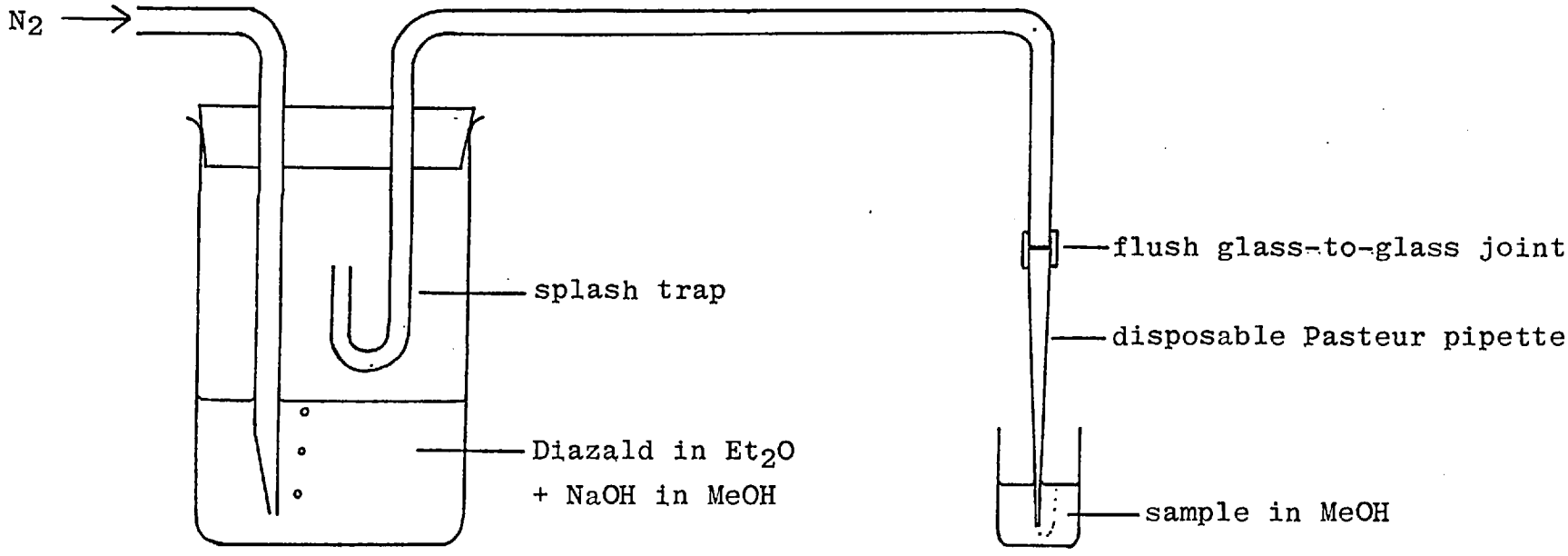
Production of Mass Spectra.

Prior to mass spectrometric analysis, taurine conjugates were derivatized to their methyl esters using diazomethane. The diazomethane was generated from Diazald® (*N*-methyl-*N*-nitroso-4-toluene sulphonamide, Aldrich Chemical Company, Inc., U.K.) by the action of methanolic NaOH.

To a saturated solution of Diazald in diethyl ether (25ml) was added a saturated solution of NaOH in MeOH (25ml). The diazomethane-diethyl ether azeotrope was produced and bubbled through a solution of the metabolite (5mg) in MeOH (5ml). The reaction was stopped when the yellow colour of diazomethane had persisted and the evolution of small bubbles of nitrogen in the sample solution had ceased. The apparatus contained no ground-glass joints (see following Fig.), since diazomethane is reported to be explosive on contact with such surfaces (Gutsche, 1954). After use, the apparatus was carefully washed with glacial acetic acid to destroy diazomethane.

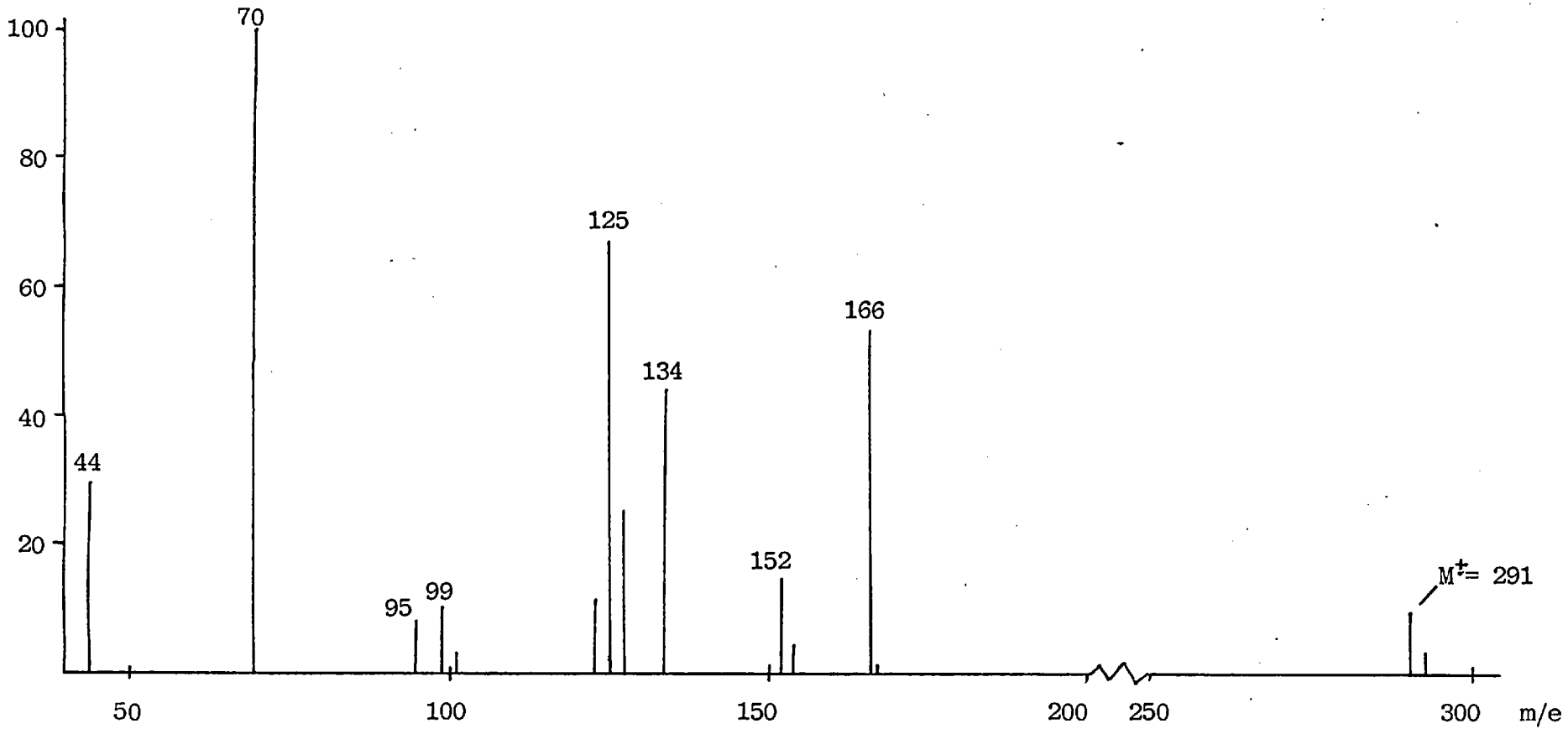
The sample (1µl=1µg) was submitted to mass spectrometric analysis in a Varian MAT CH5 Mass Spectrometer by the use of a direct insertion probe. Ionisation energy was 70eV and the source temperature was between 50 and 250°C. Mass spectra shown here are reproduced from U.V. traces and have been simplified to show only the major ions.

Ground-glass-free Diazomethane Generator for the Methylation of Samples for Mass Spectrometry.

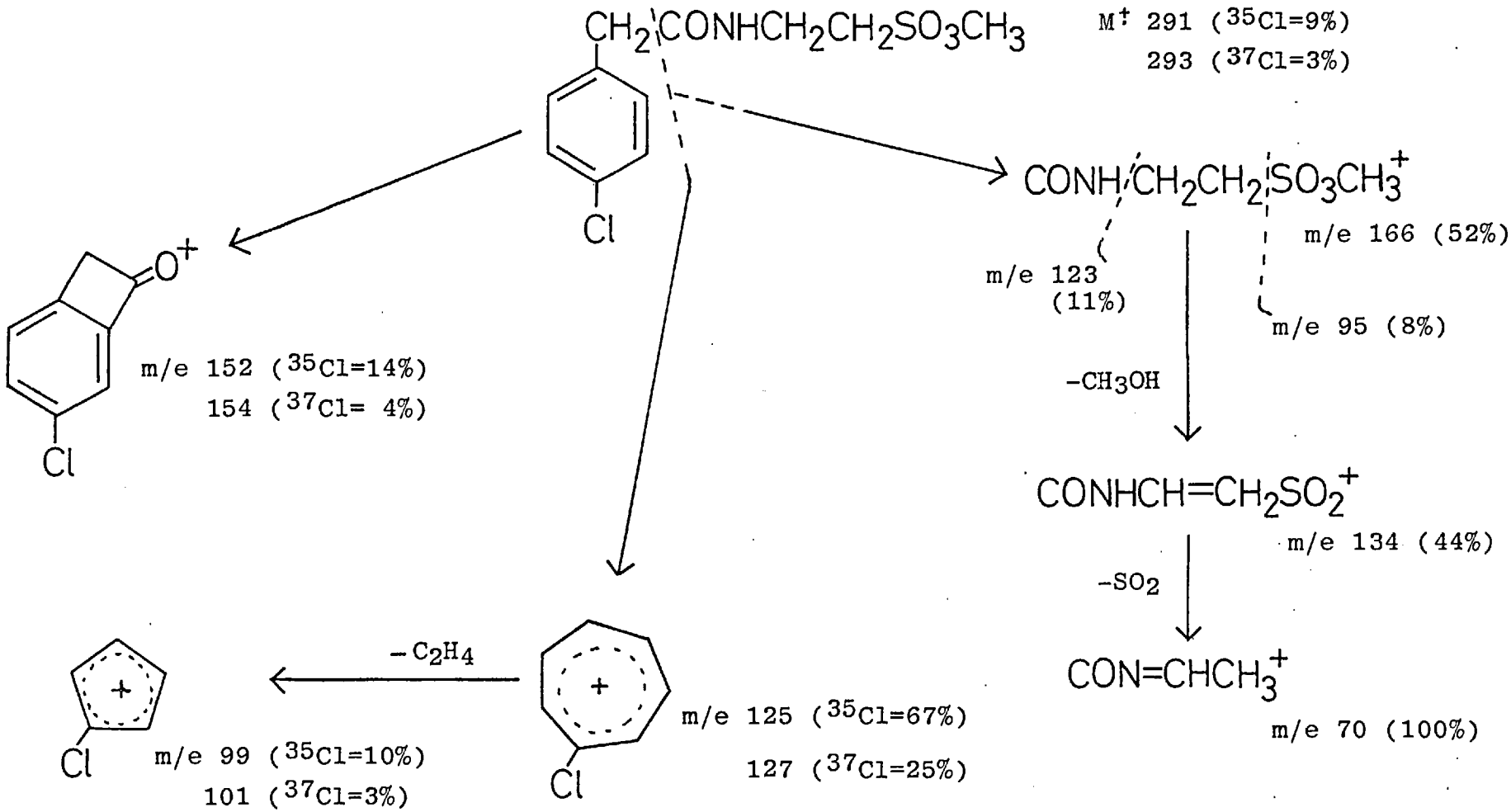


Mass Spectrum of 4-Chlorophenacetyltaurine Methyl Ester.

(120°C)

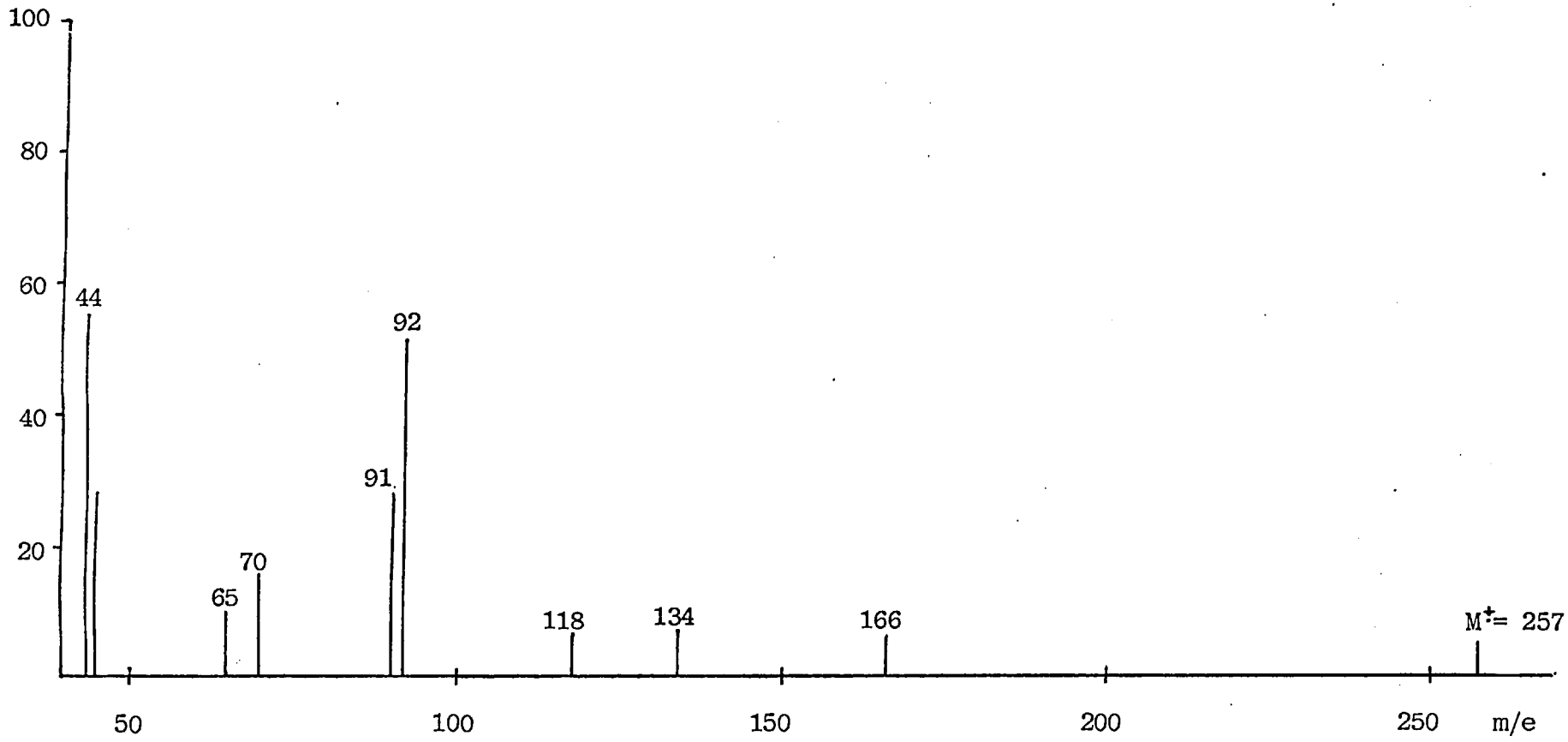


Proposed Fragmentation of 4-Chlorophenacetyltaurine Methyl Ester.

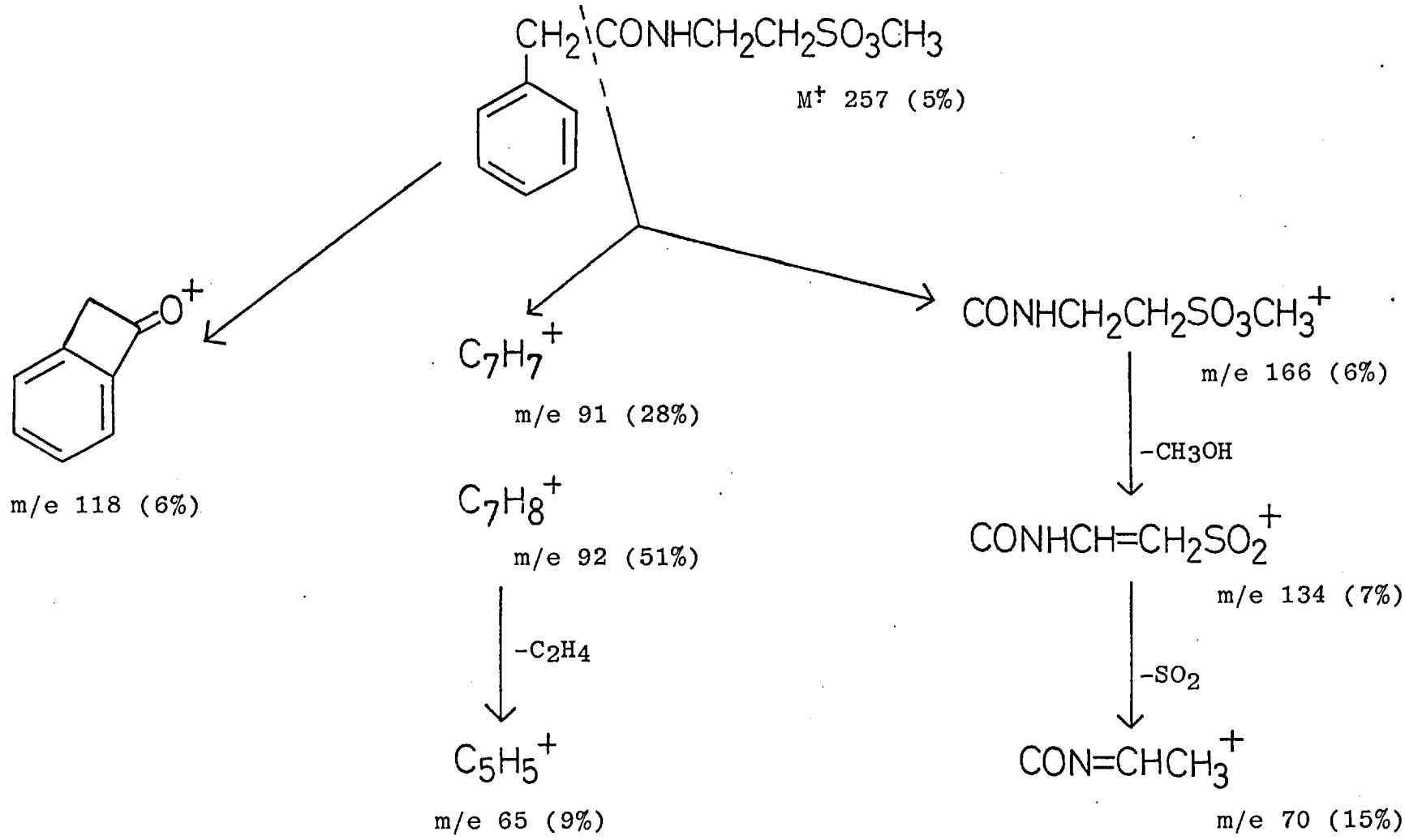


Mass Spectrum of Phenacetyltaurine Methyl Ester.

(75°C)



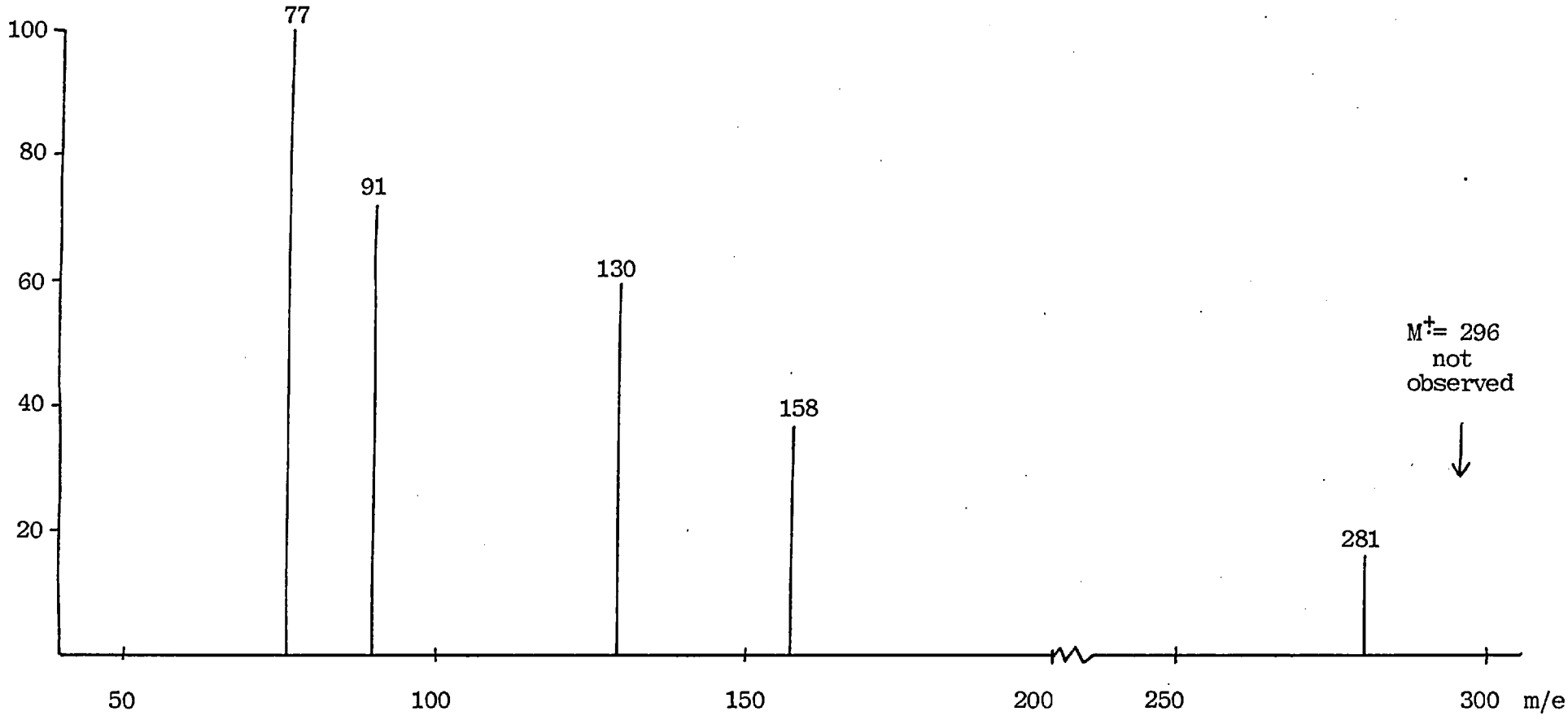
Proposed Fragmentation of Phenacetyltaurine Methyl Ester.



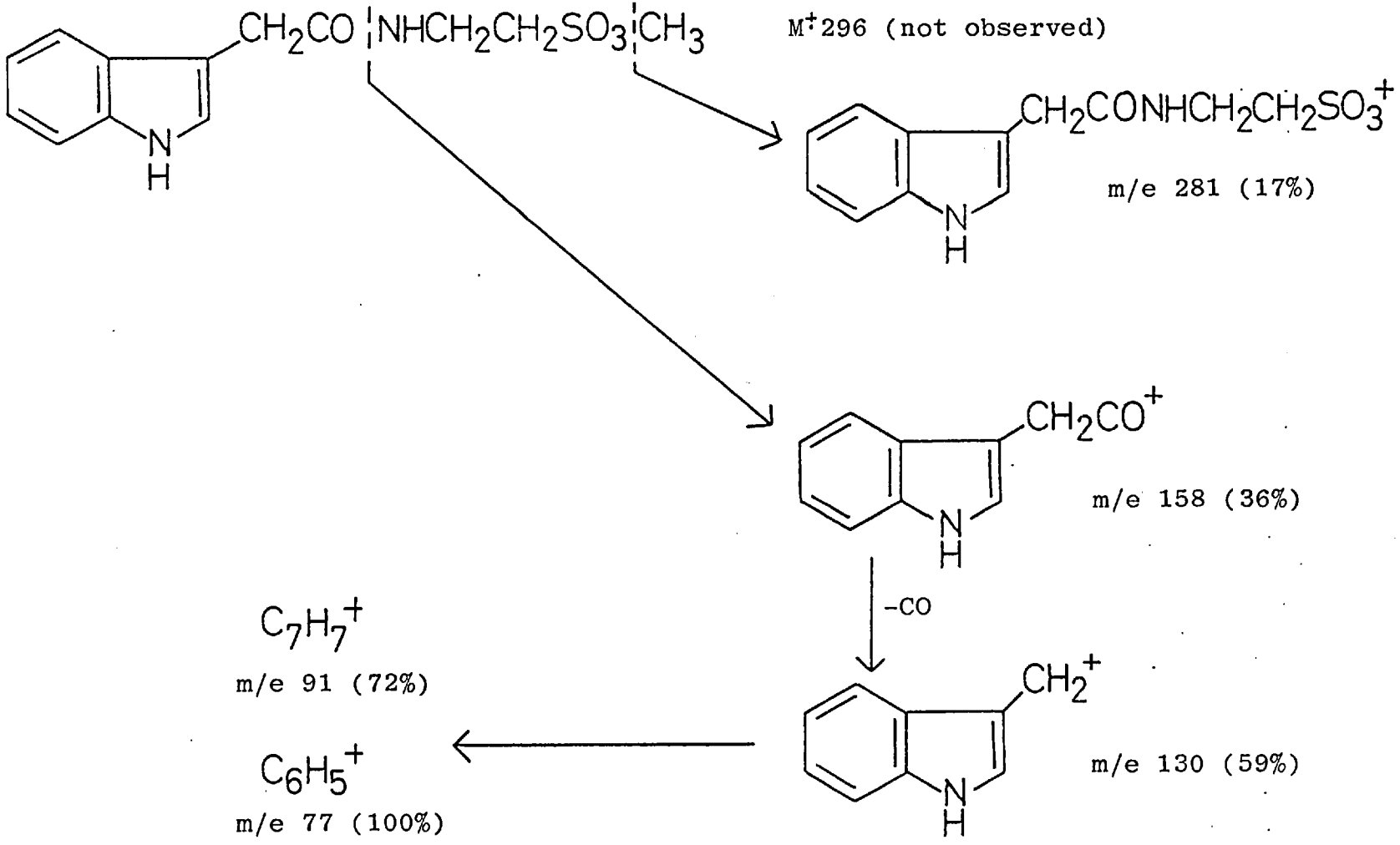
$16^+, 17^+, 28^+, 32^+ = 100\%$

Mass Spectrum of Indol-3-ylacetyltaurine Methyl Ester.

(220°C)

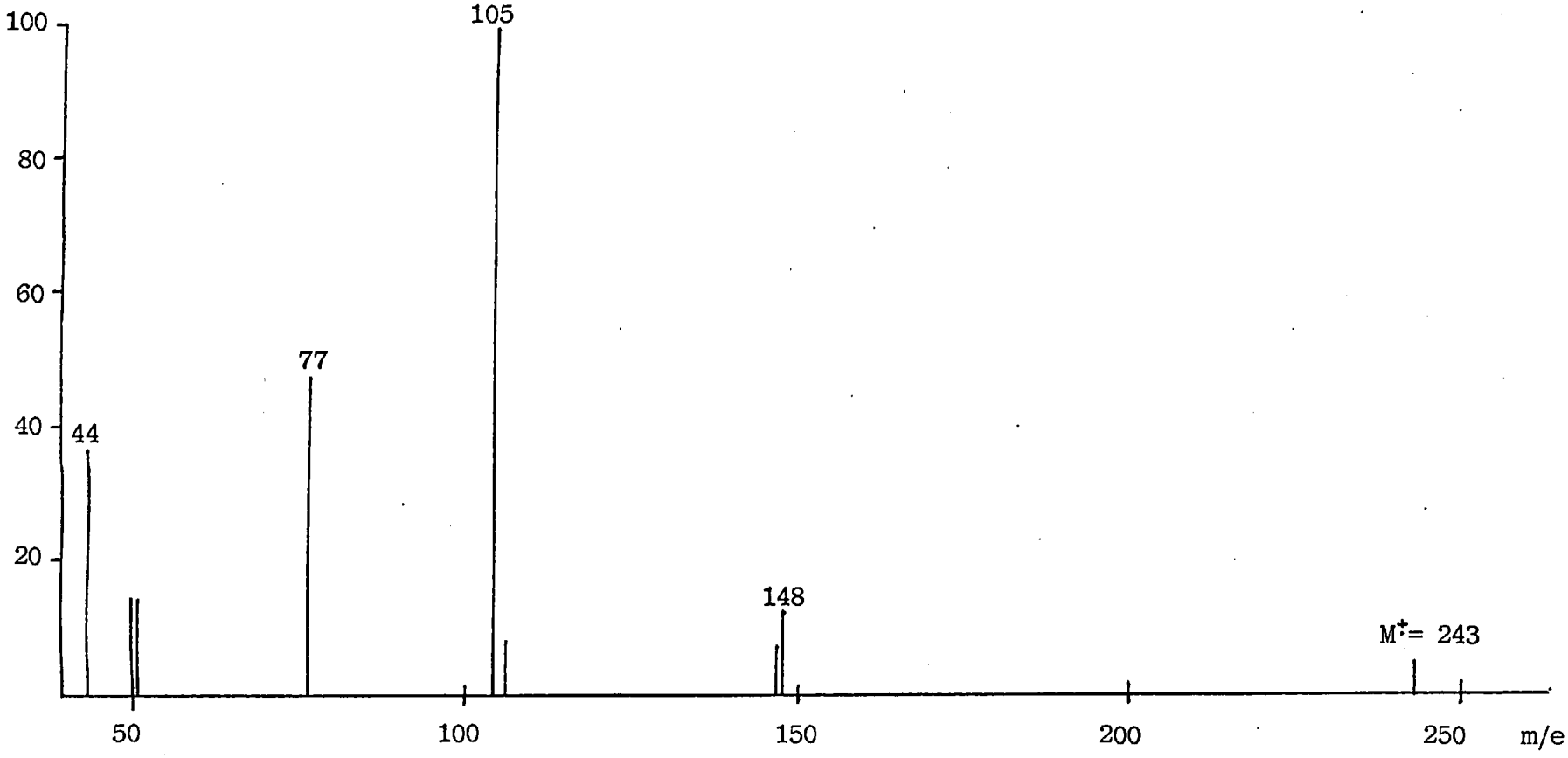


Proposed Fragmentation of Indol-3-ylacetyltaurine Methyl Ester.

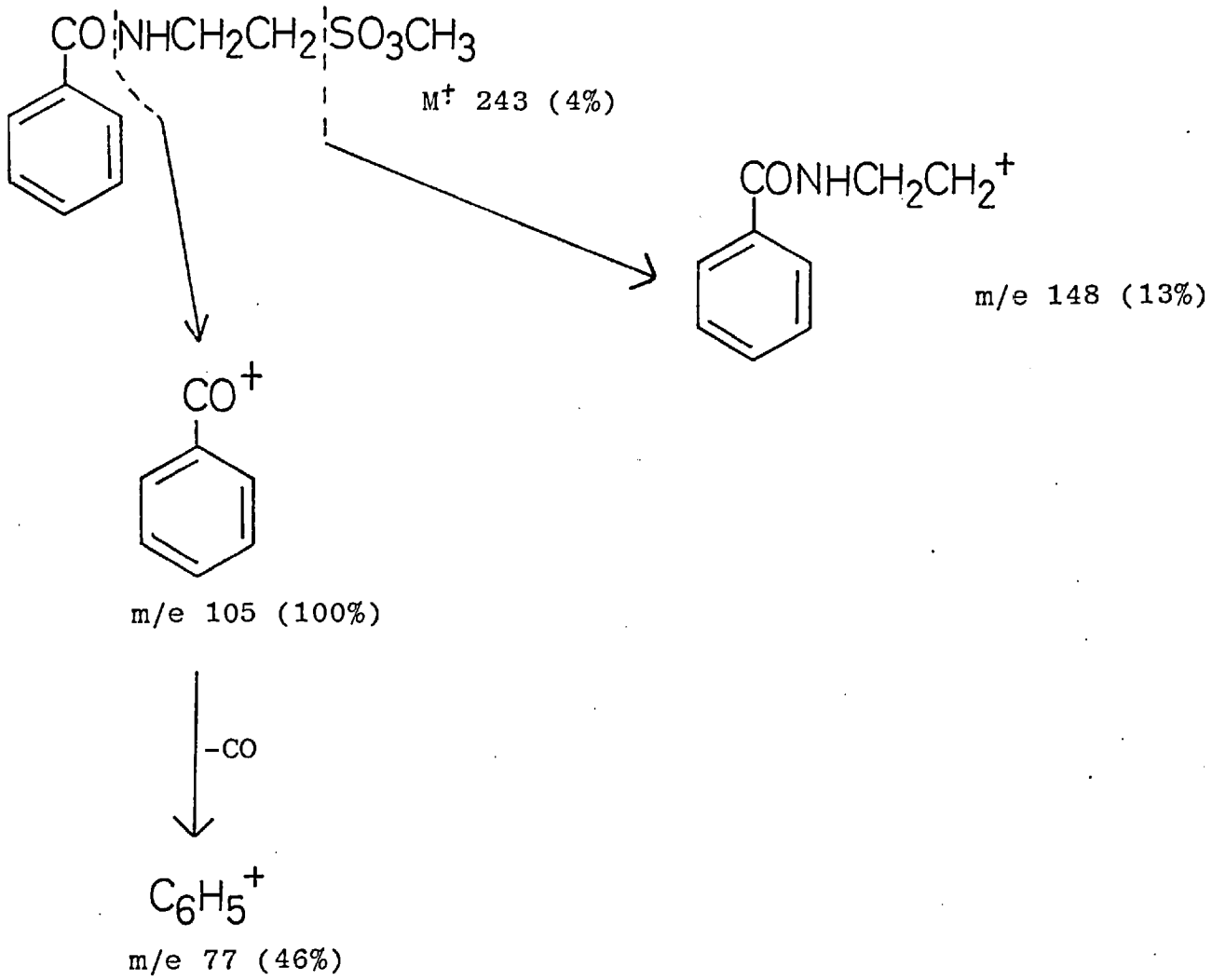


Mass Spectrum of Benzoyltaurine Methyl Ester.

(135°C)



Proposed Fragmentation Of Benzoyltaurine Methyl Ester.



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